Nickel-Superoxiddismutasen von schwermetallresistenten Streptomyceten

Dissertation
zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller- Universität Jena

von Dipl. Biol. Astrid Schmidt

geboren am 8. Juli 1977 in Suhl/ Thüringen

Datum der Verteidigung: 28.03.2011

Gutachter: 1. Prof. Dr. Erika Kothe
2. Prof. Dr. Gabriele Diekert
3. Prof. Dr. James Weston

Inhaltsverzeichnis

1. Einleitung

1.1 Streptomyceten: Lebensweise und Vorkommen

Streptomyceten sind Gram positive filamentöse Actinobakterien, deren vegetatives Myzel während der Koloniebildung morphologischer Differenzierung unterliegt (Jakimowicz, 2007, Claessen *et al.*, 2006). In einem komplex regulierten Zellzyklus multizellulärer Art wird unter ungünstigen Wachstumsbedingungen einschließlich extrazellulärer Botenstoffe die Ausbildung von Luftmyzel und die Septierung in Sporenketten ausgelöst. Um sich dafür aus der Oberflächenspannung des Wachstumsmediums zu lösen, werden hydrophobe Proteine ausgeschieden (Flärdh und Buttner, 2009). Eine weitere wichtige Rolle spielen Zellwand-Hydrolasen, die im Vergleich zu anderen weniger polyformen Prokaryoten in großer Zahl kodiert sind (für *S. coelicolor* 56, Haiser *et al.* 2009). Geosmin und Methylisoborneol wird von Actinobakterien, Cyanobakterien und Myxobakterien abgegeben, diese Gruppen zeigen neben morphologischer Differenzierung auch die Bildung multizellulärer Komplexe (Komatsu *et al.*, 2008). Die Funktion dieser volatilen terpenoiden Metabolite ist unbekannt, weist aber auf interzelluläre Kommunikation hin.

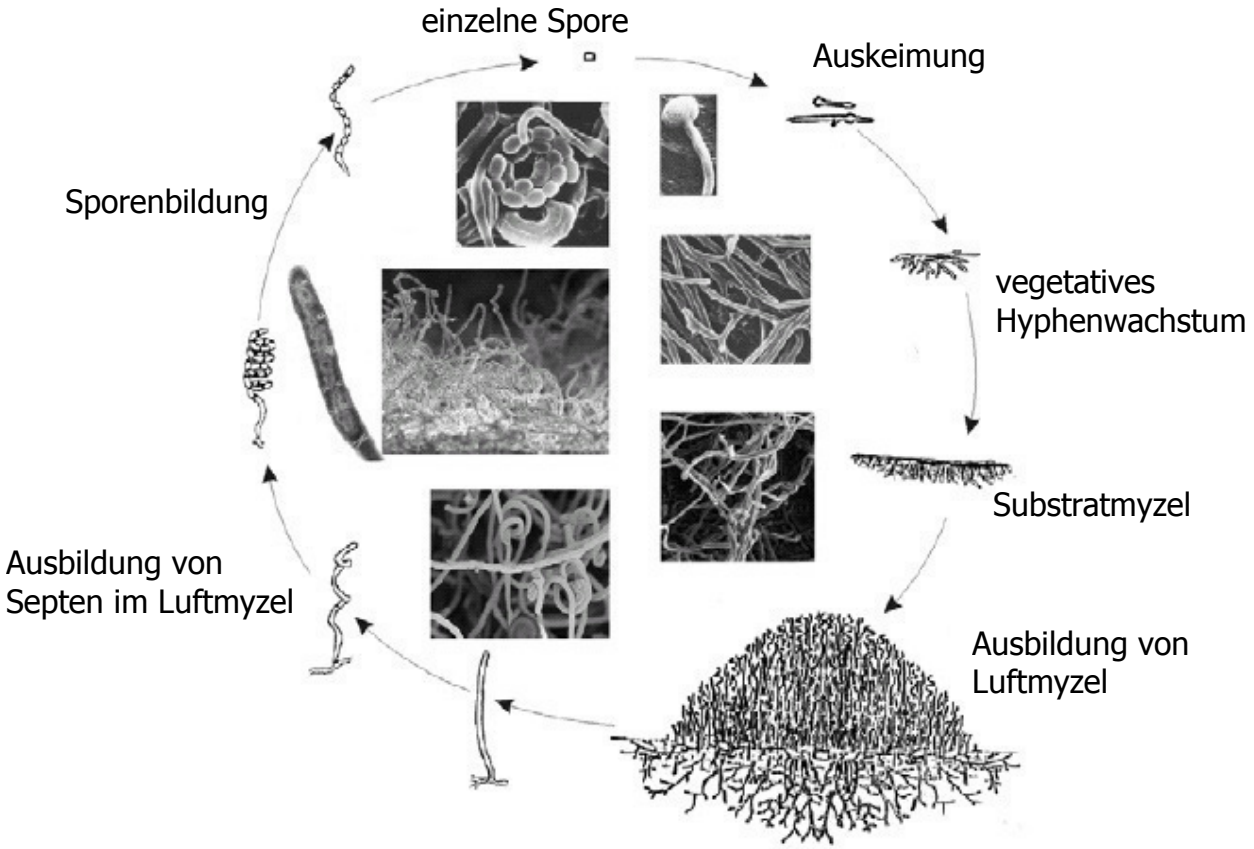

Abb. 1: Lebenszyklus der Streptomyceten (nach Jakimowicz *et al.*, 2007)

Die bodenbewohnenden Bakterien kommen sowohl terrestrisch wie auch marin vor. Terrestrisch werden Streptomyceten in der Rhizosphäre gefunden, in nährstoffarmen Böden überdauern sie als Sporen. Im Lake Mikata (Fukui, Japan) kommt es saisonal zur Blüte des toxischen Cyanobakteriums *Microcystis* (Yoshida *et al.*, 2005). Der dort aus dem Seeboden isolierte *Streptomyces grisovariabilis* NT0401 inhibiert selektiv das Wachstum von *Microcystis* (Hua *et al.* 2009). *Streptomyces sp. strain* Pol001(T) wurde aus dem im Mittelmeer lebenden Schwamm *Axinella polypoides* isoliert (Pimentel-Elardo *et al.*, 2009). Andere marine Stämme leben in der Rhizosphäre der Mangrovenwälder (Xiao *et al.*, 2009). Aus der Tiefsee wurden ebenfalls Streptomyceten isoliert, wobei angenommen wird, dass diese Organismen durch terrestrische Abrutschungen ins Meer gelangten und sich dort an Druck und andere Bedingungen der Tiefsee (in 300-10900m Tiefe) angepasst haben (Colquhoun, 1998).
Ihre Anpassungsfähigkeit und Vielfältigkeit ist unter anderem auf den Besitz von Plasmiden zurückzuführen, die zwischen verschiedenen Stämmen ausgetauscht werden können (horizontaler Transfer). Auch die Resistenz vieler Streptomyceten gegenüber Schwermetallen ist, soweit bisher untersucht, häufig plasmidkodiert. Mehr als 7000 verschiedene Sekundärmetabolite wurden in Streptomyceten bereits gefunden (Chater *et al.*, 2010). Neben der Ausscheidung substratbindender Proteine und hydrolytischer Enzyme zum Abbau von Chitin und Cellulose, stehen sie in besonderem Fokus bei der Suche nach weiteren Antibiotika. Amphipathische Proteine für die Anheftungsfähigkeit der Hyphen, Geosmin und Methylisoborneol und verschiedene Siderophortypen (Eisenassimilation) werden von den meisten Streptomyceten ausgeschieden. Ihre Omnipotenz und die große Zahl exkretierter Sekundärmetabolite beeinflusst ihre gesamte benachbarte Mikroflora. Neuere Studien geben Hinweise auf eine atmosphärische Beeinflussung durch die Aufnahme des indirekt als Treibhausgas wirkenden Wasserstoffmoleküls. Constant *et al.* (2008) isolierten einen bodenbewohnenden *Streptomyces sp.* PCB7, der eine hohe Affinität für H_2 aufweist. Der nickelabhängige Anstieg der H_2-Aufnahme weist möglicherweise auf die Beteiligung einer NiFe-Hydrogenase an dem noch unbekannten Mechanismus hin.

1.2 Superoxid-Dismutasen

Sauerstoff ist zum einen für aerobe Organismen lebensnotwendig, zum anderen können durch seine starke Oxidationsbereitschaft leicht hochreaktive Sauerstoffspezies entstehen, die wiederum verschiedene Zellbestandteile oxidieren und damit schädigen können. Vor allem Peroxide (wie H_2O_2), das Superoxidanion (O_2^-) und das am stärksten toxische Hydroxylradikal ($OH^{\cdot}$) spielen dabei eine Rolle. Aerobe Organismen haben dagegen verschiedenste Abwehrmechanismen entwickelt. Neben der Pigmentierung (z.B. Carotenoide) gibt es mehrere Enzyme (Katalasen, Peroxidasen, Superoxid-Dismutasen), die diese

Sauerstoffarten detoxifizieren können (Madigan *et al.*, 2000). Superoxid-Dismutasen sind strukturell und funktionell verwandte Enzyme, die katalytisch Sauerstoffradikale eliminieren. Sie sind Schlüsselenzyme zum Schutz der Zellen vor Sauerstoff-Stress und essentiell für aerobe Organismen. Der Name des Enzyms deutet darauf hin, das ein Superoxidanion oxidiert, das andere gleichzeitig reduziert wird: $2\ O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$. Wasserstoffperoxid wird anschließend durch Katalasen bzw. Peroxidasen eliminiert (Fridovich, 1995).

Unterschiede weisen die Superoxid-Dismutasen in der Besetzung ihrer Metall-Cofaktoren auf. Neben Kupfer, Eisen und Mangan wurde Nickel als Cofaktor für Superoxid-Dismutasen gefunden (Youn *et al.*, 1996a). Anhand ihrer Eigenschaften werden diese Enzyme in drei Gruppen unterteilt.

Die CuZnSOD ist vor allem im Cytosol der Eukaryoten zu finden. Für Prokaryoten wurde das erste Enzym bei *Photobacterium leiognathi* gefunden, wobei zunächst angenommen wurde, dass das Gen aus dem eukaryotischen Wirt (Ponyfisch) stammt (Dunlap und Steinman, 1986). Aber auch frei lebende Prokaryoten besitzen CuZnSOD; periplasmatisch aktiv in gramnegativen Proteobakterien, wie *Escherichia coli* (Imlay und Imlay, 1996) und *Rhodobacter sphaeroides* (Kho *et al.*, 2004). CuZnSOD vom gleichen Typ werden auch in einigen Cyanobakterien (*Synechococcus* CC9311, Dupont *et al.*, 2008b) und mehreren Actinobakterien (*sodC*, *Mycobacterium sp.*, *Rhodococcus sp.*, *Nocardia sp.* und *Corynebacterium sp.*) gefunden, wobei die Lokalisierung dieser SOD in den Zellen noch unklar ist (deklariert als ‚periplasmatic'), eventuell könnten sie bei Cyanobakterien in den Thylakoiden wirken.

MnSOD und FeSOD werden aufgrund ihrer Ähnlichkeit in einer Gruppe zusammengefasst. Sie finden sich im Cytosol fast aller Prokaryoten, beide können auch gemeinsam in einer Zelle auftreten. MnSOD kommt überdies auch in den Mitochondrien eukaryotischer Zellen vor, was durch die Endosymbiontentheorie erklärt wird. Homologien in der Aminosäuresequenz deuten auf einen gemeinsamen evolutionären Ursprung der Mn- und FeSOD. Die FeZnSOD der Streptomyceten ist ebenfalls dieser Gruppe zuzuordnen. FeZnSOD wurden ebenfalls in *Methanobacterium bryantii*, *Thermoplasma acidophilum* und *Nocardia asteroides* (enthält FeMnZnSOD) gefunden. Priya *et al.* (2007) konnten strukturelle Unterschiede zwischen cyanobakteriellen MnSOD und FeSOD in der Cofaktorbindung zeigen.

Die NiSOD wird aufgrund ihrer Differenzen zu anderen SOD-Typen als eigene Gruppe gesehen. Sie wurde in Streptomyceten erstmalig beschrieben (Kim *et al.*, 1996, 1998b) und ist inzwischen auch für andere Organismengruppen bekannt.

Im Allgemeinen liegt die Größe der Untereinheiten bakterieller Superoxid-Dismutasen zwischen 18-22 kDa, die meist zu Dimeren und seltener zu Tetrameren zusammengesetzt

sind. Weitgehend unklar ist noch die Spezifität bei mehreren Isoenzymen in einer Zelle. So hat man für *E. coli* die Abwehr von Superoxidradikalen intrazellulärer bzw. externer Herkunft ihrer Lokalität entsprechend den cytoplasmatischen FeSOD und MnSOD bzw. der periplasmatischen CuZnSOD zuordnen können. Desweiteren soll die MnSOD effektiver in der Vermeidung vor DNA-Schäden und die FeSOD wichtiger bei aerobem Wachstum sein (Kim *et al.*, 1998b).
Einige Bakterien sind in der Lage, je nach vorhandenem Metall im Medium in das gleiche Apoenzym entweder Eisen oder Mangan einzubauen (Meier *et al.*, 1982). Diese werden kambialistische Enzyme genannt (Martin *et al.*, 1986, lat*. cambiare*: wechseln, tauschen) und wurden u.a. in *Propionibacterium shermanii*, *Streptococcus mutans* und *Bacteroides fragilis* gefunden. Sind Eisen und Mangan vorhanden, könnte die Entscheidung für eines der beiden Metalle als Cofaktor mit der Verfügbarkeit dieser Metalle in natürlichen Habitaten zusammenhängen. Unter aeroben Bedingungen liegt Eisen oxidiert vor – als Fe(III) - und ist schwer löslich, vermutlich werden deshalb andere Metalle, wie Mangan, bevorzugt verwendet (Martin *et al.*, 1986).

Streptomyces-Arten enthalten zwei Superoxid-Dismutase-Typen. Eine der Superoxid-Dismutasen enthält Eisen und Zink (FeZnSOD; Kim *et al.*, 1996). Diese Isoform tritt in den meisten Stämmen (*S. coelicolor* Müller, *S. griseus* etc.) einmal auf (kodiert von *sodF*), in *S. coelicolor* A3(2) liegen zwei Gene vor (*sodF1*, *sodF2*) (Chung *et al.*, 1999a). Daneben enthalten Streptomyceten eine NiSOD, die neben ihrem Cofaktor auch in der Aminosäuresequenz, ihren Absorptionsmaxima, der Proteingröße und ihrer Immuno-Kreuzreaktivität von anderen Superoxid-Dismutasen abweicht. Dieses Enzym kann Nickel als ökologische Nische nutzen, dessen Gehalt im Boden dem von Kupfer und Zink ähnelt. Die Fähigkeit des Nickels zur Katalyse der Superoxidanionen-Disproportionierung konnte dadurch nachgewiesen werden, dass Nickel-enthaltende Komplexe *in vitro* eine Superoxid-Dismutase-ähnliche Aktivität aufweisen (Kim *et al.*, 1996).

1.3 Eisen-Zink-Superoxid-Dismutase

FeZnSOD ist ein Homotetramer, ihre Untereinheiten besitzen eine Größe von je 22,2 kDa mit 0,36 mol Fe und 0,26 mol Zn pro Untereinheit. Die Aminosäuresequenz ähnelt der anderer FeSOD und MnSOD, besonders von *Propionibacterium shermanii* und *Mycobacterium spp.* Die Transkription beginnt 38 Nukleotide vor dem Startcodon. Eine mögliche Ribosomenbindestelle liegt bei –9 (GGAGG) vor dem Translationsstart. Die Sequenz TAGCGT liegt in der –10-Region und ähnelt dem Consensus, die von dem vegetativen Sigmafaktor σ^{hrdB} von *S. coelicolor* erkannt wird (TAGAPuT). An der –35-Region wurden dagegen keine

Ähnlichkeiten zu anderen Promotorsequenzen gefunden (Kim *et al.*, 1998b für *S. coelicolor* Müller). Das Gen *sodF* ist im Stamm *S. coelicolor* A3(2) dupliziert vorhanden. Die Nukleotidsequenzen der beiden Kopien *sodF1* und *sodF2* sind zu 100% bzw. 88% mit *sodF* von *S. coelicolor* Müller identisch (Chung *et al.*, 1999b).

1.4 Nickel-Superoxid-Dismutase

Das Gen *sodN* in Streptomyceten kodiert für 131 Aminosäuren. Zur Aktivierung wird das Präprotein um 14 Aminosäuren vor dem N-terminalen Histidin prozessiert. Das reife Apoprotein (Monomer), besteht aus 117 Aminosäuren (13,4 kDa). Nickel ist katalytischer Cofaktor und spielt außerdem in der Regulation der Transkription und in der Prozessierung zum Holoenzym eine Rolle. Das aktive Enzym ist ein Hexamer mit je einem Nickelliganden pro Untereinheit (Wuerges *et al.*, 2004, Bryngelson *et al.*, 2004).

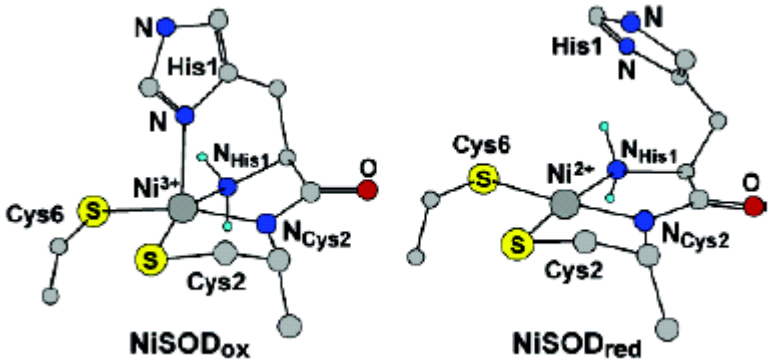

Abb. 2: Das aktive Zentrum der NiSOD im oxidierten (links) und reduzierten Zustand (aus Fiedler und Brunold, 2007)

Die Struktur der NiSOD ist unter den Superoxid-Dismutasen einzigartig in der Bindung des Cofaktors über Cysteinreste (S-Liganden von C2 und C6) und im Aufbaus als Hexamer (Ryan *et al.*, 2010). Im oxidierten Zustand ist Ni^{3+} innerhalb einer quadratischen Pyramide koordiniert: der Imidazolring des H1-Restes dient als axialer N-Ligand, die Standfläche wird durch zwei Thiolate von C2 und C6, der deprotonierten Amidgruppe von C2 und der N-terminalen Aminogruppe von H1 gebildet. Bei der Oxidation des Superoxidanions dissoziiert der axiale Ligand, somit ist Ni^{2+} nur planar koordiniert (Fiedler und Brunold, 2007). Die für SOD untypische Nutzung von Cys-Liganden wird auch für andere Nickelenzyme als essentiell für die Absenkung des Redoxpotentials der Nickelzentren beschrieben. Die Proteinumgebung stellt außer dem Abgleichen des Redoxpotentials (~290mV für NiSOD) die Bereitstellung von Protonen und die sichere Zuleitung von Anionen sicher (Herbst *et al.*, 2009). Das vollständig oxidierte Hexamer besteht aus einer Mischung von 50:50 Ni(II)/ Ni(III). Dabei spielt

möglicherweise der axiale H1-Ligand eine Rolle, für die Balance der Zustände und die Kommunikation der Nickelzentren sind weitere Forschungen nötig. Eventuell spielen Wasser-vermittelte Wasserstoffbrücken (H53) zwischen den Monomeren eine Rolle (Herbst *et al.*, 2009).

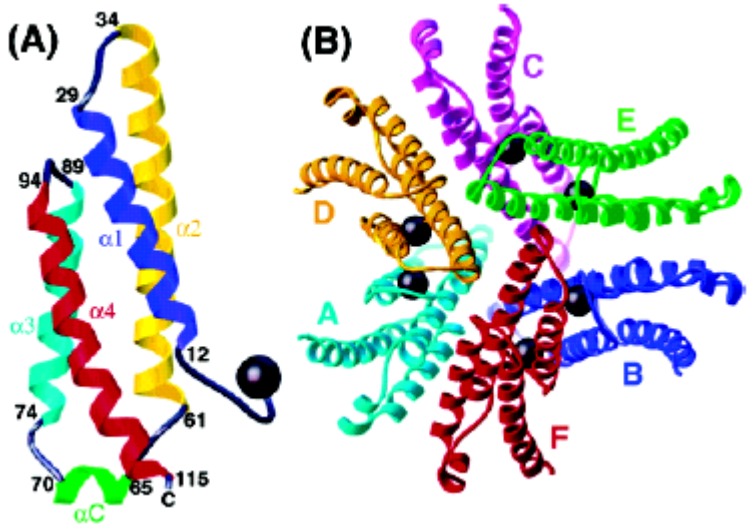

Abb. 3: NiSOD-Untereinheit (A) und Hexamer (B), Nickel ist schwarz markiert (aus Barondeau *et al.* 2004)

Im nativen Zustand sind die Monomere mittels vier alpha-Helices (α1-4) mit einer kurzen Verbindungshelix αC gefaltet, wobei die ersten N-terminalen Aminosäurereste einen Haken bilden, in dem Nickel koordiniert wird (H1, C2, C6). An der Außenseite der Monomere finden sich anders als erwartet vorwiegend polare und geladene Seitenketten, hydrophobe Bereiche für die multimere Assemblierung fehlen scheinbar. Die NiSOD tritt aber als Hexamer mit sechs identischen Untereinheiten auf, wobei jeweils drei Monomere wie die Beine eines dreibeinigen Stativs angeordnet sind. Beide Trimere berühren sich über die Verbindungshelices (αC, „Fuß" der Untereinheit). Zur Stabilisierung tragen Ionenbindungen, hydrophobe und Wasser-vermittelte Wasserstoffbrücken bei (Barondeau *et al.*, 2004). Das Protein ist sehr stabil, Erwärmung bis zu 70°C und pH-Schwankungen zwischen 4,0 und 8,0 ändern wenig an seiner Aktivität (Youn *et al.*, 1996a).

1.5 Regulation der Superoxid-Dismutasen-Synthese

Das Gen *sodN* der Streptomyceten besteht aus 393 Nukleotiden, der Transkriptionsstartpunkt befindet sich 30 Nukleotide vor dem Startcodon. Eine mögliche Ribosomenbindestelle liegt an Position –9 (AGGAAG) vor dem Translationsstart (Kim *et al.*, 1998a). Consensus-Promotorsequenzen für verschiedene Sigmafaktoren von *S. coelicolor*

(σ^{hrdB}, σ^{hrdD}, σ^{sigE} und σ^{31}) konnten nicht identifiziert werden, die Sequenz wird demnach entweder von einem anderen Sigmafaktor erkannt oder zur Transkription ist zusätzlich ein Aktivierungsfaktor notwendig. Die Transkriptionstermination liegt 76 Nukleotide hinter dem Stoppcodon; *sodN* kodiert also für ein monocistronisches Transkript (Kim *et al.*, 1998a). Durch weitere Forschungsarbeiten hat sich herausgestellt, dass es innerhalb der Gattung *Streptomyces* verschiedene Regulationswege für die *sodF*- und *sodN*-Transkription gibt.

In allen untersuchten *Streptomyces*-Arten wird die Synthese der FeSOD und der NiSOD in Abhängigkeit von Nickel gegensätzlich kontrolliert (Kim *et al.*, 1996). Untersuchungen zur Nickelkonzentration, bei der *sodN* stimuliert bzw. *sodF* gehemmt wird, wurden in verschiedenen Vollmedien ausgeführt, Chung *et al.* (1999a) nutzten NA-Agarplatten, während Kim *et al.* (1998a) flüssiges YEME-Medium verwendeten. Dabei wurde die Synthese der NiSOD von *S. coelicolor* Müller durch 200 µM $NiCl_2$ (Kim *et al.*, 1998a) stimuliert und FeZnSOD von *S. coelicolor* Müller und FeZnSOD1 von *S. coelicolor* A3(2) bereits bei 0,5 µM $NiCl_2$ bzw. FeZnSOD2 von *S. coelicolor* A3(2) bei 10 µM vollständig gehemmt (Chung *et al.*, 1999a). Der Antagonismus ist dennoch nicht absolut, da unter verschiedenen Einflüssen auch beide Superoxid-Dismutasen vorkommen. Bei geringsten Nickel-Spuren wird die NiSOD bereits induziert, die FeZnSOD dagegen erst bei höheren Ni-Konzentrationen gehemmt (Kim *et al.*, 1996, 1998b).

SrnR/Q von *S. coelicolor* A3(2) und *S. griseus*

Die Regulation von *sodN* und *sodF* durch Nickel auf Transkriptionsebene bedingt nickelsensitive Transkriptionsregulatoren. Chung *et al.* (1999a) fanden für *sodF1* von *S. coelicolor* A3(2) Promotorelemente, die mit einem durch Nickel aktivierten Bindeprotein negativ reguliert werden. Diese *cis*-aktivierte Region befindet sich im Bereich –60 bis +30 um den Transkriptionsstart. Kim *et al.* (2000) identifizierten diese Region bei –2 bis +15 der *sodF* von *S. griseus*. In diesem Bereich findet sich eine invertierte Sequenzwiederholung (+1 TTGCA; +11 TGCAA), die möglicherweise bei der Bindung eines nickelsensitiven Repressors eine Rolle spielen könnte. Ähnliche Dyadensymmetrien wurden in Promotoren des *nik*-Operons und der *sodA* (FeSOD) von *E. coli* sowie der *sodA* von *Bacillus subtilis* gefunden, was nach Kim *et al.* (2000) eindeutig für einen Operator spricht, der an einer nickelvermittelten Transkriptionshemmung von *sodF* beteiligt ist.

Diese Vermutung wurde durch Kim *et al.* (2003b) bestätigt. Direkt hinter *sodF* von *S. griseus* wurden zwei ORFs mit überlappenden Stoppcodon von *srnR* und Startcodon von *srnQ* (vermutlich um 1:1-Stöchiometrie zu erreichen) in gleicher Leserichtung wie *sodF* gefunden, die an der Repression dieser FeSOD beteiligt sind. Allerdings ändert jedes Protein für sich

nichts an der *sodF*-Expression. Das Gen für SrnR (*sodF* repression by nickel repressor) beginnt 160 bp hinter dem Stoppcodon von *sodF* und kodiert für ein Protein mit DNA-Bindemotiv (Helix-Turn-Helix), das unter anderem Transkriptionsregulatoren der ArsR-Familie ähnelt (Kim *et al.*, 2003b). Das Gen für SrnQ (Co-Repressor) startet 501 bp downstream von *sodF*, die abgeleitete Aminosäuresequenz ähnelt keinem bekannten Protein, enthält aber 26% Arginin. Beide Proteine lagern sich unabhängig von Nickel wahrscheinlich zu einem Oktamer aus je vier Untereinheiten zusammen. SrnQ ändert durch Nickelbindung (1 Ni^{2+}/Molekül) seine Konformation und es wird postuliert, das sich daraufhin die DNA-Bindeaktivität von SrnR erhöht (Abb. 4, Kim *et al.*, 2003b). Daraufhin kann der Komplex an die inverted repeat-Struktur im *sodF*-Promotor (TT***G***CAN7TGCAA, mit ***G*** an +1) binden und unterdrückt damit die *sodF*-Synthese. In *S. coelicolor* A3(2) M145 zeigt ein putatives ArsR-kodierendes Gen 52% Ähnlichkeit zu SrnR (Kim *et al.*, 2003b), was einen ähnlichen Mechanismus vermuten lässt (siehe auch Chung *et al.*, 1999a).

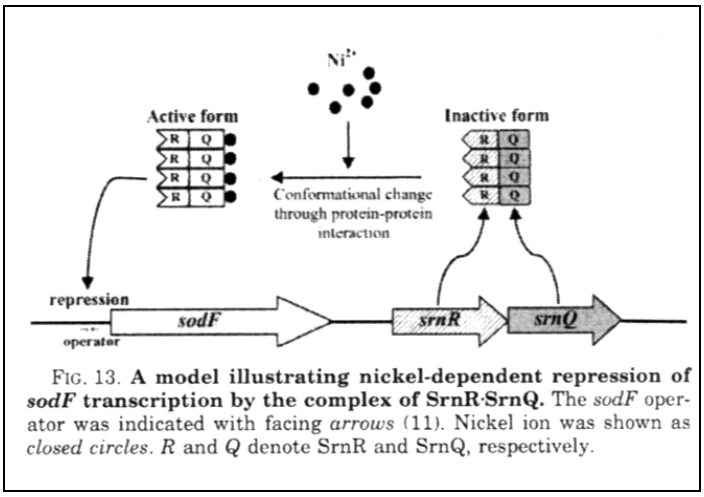

FIG. 13. **A model illustrating nickel-dependent repression of *sodF* transcription by the complex of SrnR·SrnQ.** The *sodF* operator was indicated with facing *arrows* (11). Nickel ion was shown as *closed circles*. *R* and *Q* denote SrnR and SrnQ, respectively.

Abb. 4: Modell zur Illustration der nickelabhängigen Repression der *sodF*-Transkription durch den Komplex SrnR-SrnQ in *S. griseus* (aus Kim *et al.*, 2003b)

<u>Nur von *S. coelicolor* Müller und *S. avermitilis*</u>

In *S. coelicolor* Müller hingegen finden sich keine SrnR/Q-Homologe im Genom. Stattdessen wurde ein zur Fur-Familie gehörendes Protein Nur gefunden, dass nickelabhängig an den *sodF*-Promotor bindet und als Repressor wirkt (Ahn *et al.*, 2006). Nur agiert als Homodimer, mit einer von Fur abweichenden DNA-Bindedomäne und zwei Metallbindestellen: einer unspezifischen Zn- bzw. Ni-Bindung und einer spezifischen Ni-Bindestelle (*S. coelicolor* A3(2), An *et al.*, 2008, 2009). Nur und die flankierenden ORFs (unknown proteins) mit gleicher Orientierung sind auch bei *S. avermitilis* vorhanden (Ahn *et al.*, 2006). Gleichzeitig reguliert Nur auch das Nickel-Transportsystem negativ, in dem es an den *nikA*-Promotor des

NikABCDE-Operons bindet (Ahn *et al.*, 2006). Eine direkte Interaktion von Nur mit dem *sodN*-Promotor konnte nicht bestätigt werden. Für *E. coli* ist eine positive Regulation von *sodB* (FeSOD) durch Fur (ferric uptake regulation protein) bekannt (Compan und Touati, 1993).

1.6 Nickel und NiSOD-Prozessierung

Das Wachstum von *Streptomyces*-Arten in Nickel-haltigen Medien induziert schon in geringsten Spuren die NiSOD-Synthese (Kim *et al.*, 1996, u.a.). Nickel ändert nicht die Stabilität der *sodN*-mRNA, demnach induziert Nickel die *sodN*-Transkription und beeinflusst nicht das Transkript (Kim *et al.*, 1998a, b). Gleichzeitig wird die FeSOD-Synthese durch nickelabhängige Repressoren gehemmt (SrnR/Q bzw. Nur). Die Regulation auf Transkriptionsebene ermöglicht eine schnelle Antwort, um die Gesamtmenge der Superoxid-Dismutasen pro Zelle zu optimieren (Chung *et al.*, 1999a).

Nickelvermittelte Prozessierung und Oligomerisierung der NiSOD

Kim *et al.* (1998a) exprimierten *sodN* aus *S. coelicolor* Müller homolog in *S. lividans* TK24 und heterolog in *E. coli* DH5α. In *S. lividans* TK24 wurden daraufhin wie in *S. coelicolor* Müller zwei SOD-Aktivitäten mit entsprechender Regulation durch Nickel gefunden. NiSOD wird in diesem System in 20facher Menge produziert, wobei bei fehlendem Nickel größere Mengen als Apoproteine (NiSOD-Monomere) liegen bleiben, die erst nach Nickel-Zugabe in aktive Proteine umgewandelt werden. Für *E. coli* wurden Vektoren mit dem vollständigen Konstrukt bzw. fehlendem 14 AA-N-Terminus eingesetzt. In beiden Fällen konnte nur inaktive NiSOD gefunden werden. Erst nach Zugabe von Nickel in das Medium wurde für den Vektor mit unvollständigem *sodN*-Gen aktives Protein nachgewiesen, jedoch nur in geringem Maße. Da Nickel unspezifisch durch das Mg-Transportsystem in die Zelle gelangen kann, fehlen *E. coli* möglicherweise die an der NiSOD-Faltung und -Prozessierung beteiligten Proteine (Kim *et al.*, 1998a).

Direkt benachbart zum *sodN*-Gen finden sich bei Streptomyceten und Cyanobakterien Gene der Serinprotease-Familie (*orfX*/ *sodX*). Eitinger (2004) exprimierte *sodN* von *Prochlorococcus marinus* MIT9313 parallel mit dem downstream liegenden *sodX* in *E. coli*. Nach Wachstum auf Nickel konnte er katalytisch aktive NiSOD nachweisen, vermutlich prozessiert *sodX* die cyanobakterielle NiSOD-Vorstufe N-terminal.

Nickeleinbau in Nickelenzyme

Nickel-Enzyme sind eine relativ seltene Klasse der Metalloenzyme. Neben der Nickel-Superoxiddismutase ist dieser Ligand in Hydrogenasen, Kohlenmonoxiddehydrogenase, Urease, Glyoxylase I, cis-trans-Isomerase, Coenzym F430 und der Methylreduktase gefunden worden (Watt und Ludden, 1999). Inzwischen sind weitere Proteine, wie Nur oder NikR bekannt, die Nickel koordinieren (An *et al.*, 2009, Dosanjh und Michel, 2006, Kaluarachchi *et al.*, 2010). Um das Metall einzubauen, muss es Nickel-prozessierende Systeme geben, die für Erkennung, Transport und Einbau von Nickel verantwortlich sind. In Streptomyceten wurden noch keine direkt an der NiSOD-Synthese beteiligten Proteine identifiziert. Für den Import von Ni^{2+} in die Zelle sind neben dem unspezifischen Transport durch Mg^{2+}-Transport-Systeme (Watt und Ludden, 1999) hochaffine Nickeltransporter verantwortlich, die von Ahn *et al.* (2006) auch in *S. coelicolor* gezeigt wurden. In der Zelle wird Ni^{2+} an Nickel-Bindeproteine assoziiert, die den Schutz vor freiem Nickel und Transport zu den Apo-Nickelenzymen bewirken. Für die Insertion des Ni^{2+} sind Proteine mit Nukleotid-Bindeschleifen (P-Loop) und histidinreichen Regionen (Nickel-Bindedomäne) identifiziert worden. Außerdem sind Chaperone beteiligt, die das Apoenzym in der Konformation halten, die den Nickeleinbau erlaubt (Watt und Ludden, 1999). Der Einbau des Nickels und dessen feste Verankerung im Enzym muss sicherstellen, dass die Reaktionen des Nickels im Reaktionszentrum kontrolliert ablaufen können. In den NiFe-Hydrogenase-Operons von *E. coli* wurden Endopeptidasen gefunden, die das Apoprotein nach dem Einbau von Nickel C-terminal prozessieren und die Cofaktoren somit in das Protein einschließen (Blokesch *et al.*, 2002).

In *S. seoulensis* wurde von Kim *et al.* (2003a) mittels Chromatographie eine mutmaßliche Nickelchelatase CbiXhp (CbiX-homologes Protein zu metallbindender Sirohydrochlorin-Cobalt-Chelatase aus *Bacillus megaterium*) gefunden. Das Protein zeigt Ähnlichkeit zu den Cobaltchelatasen CbiX von *S. coelicolor* A3(2) (87%) und *B. megaterium* (39%). Es besitzt eine Größe von 35 kDa und 306 Aminosäurereste, und enthält neben einem histidinreichen Metallbindemotiv in der C-terminalen Region (13 Histidine von 27 Aminosäureresten) ein potentielles CXXC-Motiv, welches ebenfalls an der Metallbindung beteiligt sein könnte. Beide Motive sind in vielen bakteriellen CbiX-Proteinen enthalten. Durch Überexpression des *sodN*-Promotors gleichzeitig mit dem *cbiXhp*-Gen zeigt die NiSOD eine verstärkte Aktivität, was auf eine Beteiligung von CbiXhp als Nickelchelatase in der Synthese der NiSOD hinweisen könnte.

1.7 Ziel der Arbeit

In der Region des ehemaligen Wismutgebietes in Thüringen wurden im Bereich des Gessentals Streptomyceten mit zum Teil sehr hohen Resistenzen gegenüber verschiedenen Schwermetallen isoliert (Amoroso *et al.*, 2000, Schmidt *et al.*, 2007, 2009), deren intrazelluläre und extrazelluläre Resistenzmechanismen untersucht werden sollten (Schmidt *et al.*, 2010, Kothe *et al.*, 2010). Aus anderen Arbeiten wurde bekannt, dass diese Gattung ein nickelhaltiges Enzym, die NiSOD, trägt (Youn *et al.*, 1996). Da Superoxiddismutasen an der Eliminierung von durch Schwermetalle erzeugte Sauerstoffradikale beteiligt sind, stellte sich einerseits die Frage nach der Funktionalität dieses Enzyms in der Resistenzvermittlung gegenüber Schwermetallen. Aufgrund des seltenen Vorkommens nickelhaltiger Proteine, war andererseits die Isolierung des NiSOD-Gens und die NiSOD-Sequenz bei Streptomyceten von Interesse (*Schmidt *et al.*, 2007). Zum Verständnis der NiSOD-Synthese sollten Untersuchungen der Regulation der NiSOD-Synthese und nach potentiellen assoziierten Proteinen, wie hochaffine Nickeltransporter oder Nickelbindeproteinen, durchgeführt werden. Die SOD-Ausstattung und deren Homöostase in der Zelle sollten im Vergleich zu anderen NiSOD-tragenden Organismen analysiert werden. *In silico*-Analysen von Datenbankeinträgen sollten darüberhinaus zu weiteren Kenntnissen über die Verbreitung und Evolution der NiSOD führen und zur Aufklärung der Struktur und Funktion des Enzyms beitragen (*Schmidt *et al.*, 2009).

2. Übersicht zu den Manuskripten

Superoxide dismutases of heavy metal resistant Streptomycetes
Astrid Schmidt, André Schmidt, Götz Haferburg and Erika Kothe
Journal of Basic Microbiology (2007) 47, 56–62

Isolate aus schwermetallbelasteten Böden wurden auf ihre Schwermetallresistenz getestet und der am multipelsten resistente Stamm *Streptomyces acidiscabies* E13 ausgewählt. Zur Aufklärung des Resistenzmechanismus wurden die NiSOD und FeZn-Superoxid-Dismutasen des Stammes näher untersucht, das Gen *sodN* wurde sequenziert. Weiterhin wurden Cytosol sowie Medium nach dem Wachstum unter Schwermetallbedingungen auf Nickel untersucht.

Eigenanteil: Hauptanteil, Schwermetallresistenz-Tests, NiSOD-Untersuchungen unter Schwermetallstress, Sequenzierung von *sodN* aus *S. acidiscabies* E13
Mitautorenanteil: André Schmidt und Götz Haferburg (Chromatographie), Erika Kothe (Betreuung)

Heavy metal resistance in actinobacteria of the former uranium mining area near Ronneburg
André Schmidt, Götz Haferburg, Astrid Schmidt, Erika Kothe
In Proceedings des Internationalen Bergbausymposiums WISMUT 2007, Stilllegung und Revitalisierung von Bergbaustandorten zur nachhaltigen Regionalentwicklung, Wismut GmbH, 2007.

Die Ufer des Gessenbachs im ehemaligen Wismutgebiet sind durch sehr hohe Schwermetallkonzentrationen belastet. Die Bodenparameter wurden untersucht und es konnten hochresistente Mikroorganismen isoliert werden. Besonderes Interesse galt schwermetallresistenten Actinobakterien. Verschiedene Resistenzmechanismen wurden diskutiert. Neben der Untersuchung auf Plasmide konnten intrazelluläre Nickelakkumulation (*S. acidiscabies* E13) und chelatierende Substanzen (*S. tendae* F4) mit Effekt auf koexistierende sensitive Stämme gezeigt werden.

Eigenanteil: Probennahme, Versuche zur Resistenz bei *S. acidiscabies* E13
Mitautorenanteil: André Schmidt (Autor, PFGE, Chromatographie, Koresistenzversuche), Götz Haferburg (fachliche Beratung), Erika Kothe (Betreuung)

Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area

André Schmidt, Götz Haferburg, Astrid Schmidt, Ulrike Lischke, Dirk Merten, Felicia Gherghel, Georg Büchel, Erika Kothe

Chemie der Erde (2009) 69, S2, 35–44

Das Ufer des belasteten Gessenbachs im ehemaligen Uranabbaugebiet um Ronneburg/ Thüringen wurde mit besonderem Augenmerk auf Nickel auf schwermetallresistente Mikroorganismen untersucht. Das Wachstum der hoch Nickel-resistenten Stämme wurde unter weiteren Schwermetallen untersucht, unter anderem konnte Pigmentbildung beobachtet werden. Bei ausgewählten Stämme konnten Plasmide gezeigt werden.

Eigenanteil: Probennahme und Diskussion

Mitautorenanteil: Andre Schmidt (Hauptanteil), Götz Haferburg, Ulrike Lischke und Felicia Gherghel (fachliche Beratung), Dirk Merten (ICP-MS-Analytik), Georg Büchel und Erika Kothe (Betreuung)

***In silico* analysis of nickel containing superoxide dismutase evolution and regulation**

Astrid Schmidt, Matthias Gube, André Schmidt and Erika Kothe

Journal of Basic Microbiology (2009) 49, 109–118

Mittels Datenbankanalysen wurde die Verbreitung von *sodN* in weiteren Taxa, neben den bereits bekannten Trägern Streptomyceten und Cyanobakterien, untersucht. Homologe Gene wurden in verschiedensten Prokaryoten sowie in Grünalgen gefunden. Die *sodN*-Gene wurden in ihrer chromosomalen Umgebung analysiert und es konnten Rückschlüsse auf Evolution, Funktion und Regulation gezogen werden.

Eigenanteil: Hauptanteil, Datenbankanalysen und Auswertung

Mitautorenanteil: Matthias Gube (Cladogramm), André Schmidt (fachliche Beratung), Erika Kothe (Betreuung)

Screening for potential metallothioneins and metallohistins in actinobacteria
Andre Schmidt, Matthias Hagen, Eileen Schütze, Astrid Schmidt and Erika Kothe
Journal of Basic Microbiology, accepted (2010)

Alle verfügbaren Actinobakterien-Genome wurden mittels Datenbankanalyse auf potentielle Metallothioneine und Metallohistine untersucht. Von etwa 285.000 analysierten Proteinen entsprachen 103 mögliche Proteine den Kriterien von größer 15% Cys/His-Gehalt und weniger als 100 Aminosäuren. Diese Proteine wurden auf Homologien und Funktion untersucht. Des weiteren wurden Histidine als zusätzliche Metallbindedomänen, evolutionäre Herkunft und die Lokalisierung von Nachbargenen diskutiert.

Eigenanteil: Darstellung und Auswertung, fachliche Beratung
Mitautorenanteil: Andre Schmidt (Hauptanteil), Matthias Hagen (Programmierung), Eileen Schütze (Diskussion), Erika Kothe (Betreuung)

Streptomycete heavy metal resistance: Extracellular and intracellular mechanisms
Erika Kothe, Christian Dimkpa, Götz Haferburg, André Schmidt, Astrid Schmidt, and Eileen Schütze
In: Sherameti. I., Varma, A. (eds.), Soil Heavy Metals, Kapitel 10, Springer Verlag Berlin Heidelberg, ISBN 978-3-642-02436-8

Kontaminierte Böden wurden bezüglich Vitalität und Renaturierung auf überlebensfähige Organismen erforscht. Streptomyceten stellten hier eine relevante Gruppe mit hochresistenten Eigenschaften dar, die vor allem in Bezug auf Nickel weiter untersucht wurde. Extrazelluläre Resistenzmechanismen wie Chelatoren und Siderophoren, sowie intrazelluläre Einlagerung und die Funktion enttoxifizierender Enzyme, wie die Superoxiddismutase, werden zusammenfassend diskutiert.

Eigenanteil: Untersuchungen zur Resistenz und der NiSOD-Synthese, Sequenzierung des *sodN*-Gens von *S. acidiscabies* E13
Mitautorenanteil: Erika Kothe (Betreuung, Autorin), Christian Dimkpa, Götz Haferburg, André Schmidt und Eileen Schütze (Isolierung hochresistenter Stämme, Untersuchungen zu verschiedenen Resistenzmechanismen)

3. Manuskripte

3.1 Superoxide dismutases of heavy metal resistant Streptomycetes

Astrid Schmidt, André Schmidt, Götz Haferburg and Erika Kothe

Journal of Basic Microbiology (2007) 47, 56–62

Research Paper

Superoxide dismutases of heavy metal resistant streptomycetes

Astrid Schmidt, André Schmidt, Götz Haferburg and Erika Kothe

Microbial Phytopathology, Institute of Microbiology, Friedrich-Schiller-University, Jena, Germany

Heavy metal tolerant and resistant strains of streptomycetes isolated from a former uranium mining site were screened for their superoxide dismutase expression. From the strains tolerating high concentrations of different heavy metals, one was selected for its tolerance of concentrations of heavy metals (Ni, Cu, Cd, Cr, Mn, Zn, Fe). This strain, *Streptomyces acidiscabies* E13, was chosen for the purpose of superoxide dismutase analysis. Gel electrophoresis and activity staining revealed only one each of a nickel (NiSOD) and an iron (FeZnSOD) containing superoxide dismutase as shown by differential enzymatic repression studies. The gene for nickel containing superoxide dismutase, *sodN*, was cloned and sequenced from this strain. The genomic sequence shows 92.7% nucleotide identity and 96.1% amino acid identity to *sodN* of *S. coelicolor*. Expression can be activated by nickel as well as other heavy metals and active enzyme is produced in media lacking nickel but containing copper, iron or zinc. Thus, the selected strain is well suited for further characterization of the enzyme encoded by *sodN*.

Keywords: Nickel superoxide dismutase / nickel / heavy metal resistance / streptomycetes

Received: June 07, 2006; returned for modification: August 07, 2006; accepted: August 28, 2006

DOI 10.1002/jobm.200610213

Heavy metals are compounds toxic to cells, although some heavy metals, like zinc or nickel, are essential in low concentrations since they are incorporated into the active center of proteins. Excess concentrations of heavy metals, however, lead to toxicity mediated by reaction with proteins, DNA and lipids as well as by production of reactive oxygen species in the Fenton reaction catalyzed by the metal (Stohs and Bagchi 1993). Therefore, any cell exposed to heavy metal stress has to cope with reactive oxygen species including superoxide anions. These molecules are detoxified *via* superoxide dismutases (SODs) which, in two steps dimutate the superoxide to O_2 and H_2O_2 (Fridovich 1995). Subsequenly, the hydrogen peroxide is detoxified in a katalase mediated reaction.

The superoxide dismutase reaction is a redox reaction driven by an active center containing a metal which during this process alters its redox state (Barondeau *et al.* 2004). Different superoxide dimutase enzymes are known, most of which are homologous, i.e. they share sequence similarity although containing different metals: cytosolic CuZnSOD in eukaryotes and periplasmic CuZnSOD in Gram negatives; MnSOD in bacteria and mitochondria, and FeSOD in bacteria. Cambialistic SODs are known to use either iron or manganese, depending on availability of the metal (Martin *et al.* 1986). Another group of bacterial SODs contains a bimetallic center consisting of iron and zinc (FeZnSOD in actinobacteria and archaea, Kim *et al.* 1998a). An unrelated SOD without sequence similarity to the former enzymes has been described for the actinobacterial genus *Streptomyces* (Youn *et al.* 1996a, b) and in marine cyanobacteria (Palenik *et al.* 2003). Homologs to this independent enzyme have not been detected elsewhere. This enzyme contains nickel in the active center, is a homo-hexamer, and recently the structure has been published (Würges *et al.* 2004, Barondeau *et al.* 2004). Thus, in contrast to other organisms, the actinobacteria express two different superoxide dismutases in their cytosol, one containing FeZn and one with Ni in the active center. The coding sequences are non-homologous and the expression of both enzymes can be shown under laboratory culture conditions.

Correspondence: Prof. Dr. Erika Kothe, Mikrobielle Phytopathologie, Institut für Mikrobiologie, Biologisch-Pharmazeutische Fakultät, Friedrich-Schiller-Universität, Neugasse 25, D-07743 Jena, Germany
E-mail: Erika.Kothe@uni-jena.de
Fax: +49 3641 949292

A function of superoxide dismutases aside from detoxification of oxygen radicals has been described for reponse to heavy metals in the environment. Overexpression of superoxide dismutase leads to enhanced heavy metal tolerance, as shown for *Escherichia coli* or *Saccharomyces cerevisiae* (Geslin *et al.* 2001, Culotta *et al.* 1995), and to pathogenesis and cancer prevention in mammals (Leclere *et al.* 1999, Ray and Husain 2002). The essential role of the enzyme makes the study of evolution and assembly of mutations difficult. However, the evolution of two different enzymes within one organism, like *Streptomyces* containing both an iron-zinc and a nickel-containing SOD would be interesting as it might explain the metabolic versatility of streptomycetes which are known to produce many secondary metabolites, among them 80% of known antibiotics. Regulation of expression of both SODs of *Streptomyces* is described to be regulated by nickel availability. While FeZnSOD expression is repressed by the nickel sensitive complex of SrnR and SrnQ (Kim *et al.* 2003), for NiSOD a negative regulation *via* nickel sensitive repressors has been shown (Kim *et al.* 1998a, b).

For some superoxide dismutases *in vivo* and *in vitro* exchange of the central atom had been possible (see: Ciriolo *et al.* 1994, Meier 1994). This usually results in lower activities of the resulting enzyme. The influence of heavy metals on superoxide dismutases *in vivo* has not been studied in sufficient detail to allow to differentiate between heavy metal influence on the enzyme and its role in oxidative stress response.

To investigate heavy metal influence on both superoxide dismutases we screened heavy metal tolerance of actinobacterial isolates from naturally and anthropogenically nickel rich environments. The superoxide dismutases were then tested by activity staining to elucidate their response to extracellular heavy metal application.

Materials and methods

Isolation and phylogenetic analysis of actinobacteria: Isolation was performed by drying soil at 80 °C for 45 min. After resuspension in aqua dest. different dilutions were plated on minimal medium (0.5 g L-Asparagin, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7\,H_2O$, 0.01 g $FeSO_4 \cdot 7\,H_2O$, 10 g glucose, ad 1 l aqua dest.) and incubated at 28 °C for 3–7 days. Colonies of actinomycetes were inoculated on fresh plates and morphological, biochemical and genetic data (sequence of 16S rDNA) were used for identification. Strain *S. coelicolor* A3(2) (DSM 40783) was used for control.

Heavy metal measurements: 10 g of dried, ground and sieved (1 mm mesh) soil samples were resuspended in 45 ml aqua dest. and the supernatant (0.45 µm) was measured by ICP-MS (PQ3-S, Thermo Elemental). Growth supernatant or fractions after chromatography were measured by ICP-MS as well.

Heavy metal tolerance: Colonies were inoculated on plates containing minimal medium and different heavy metal concentrations (sterile filtered stock solutions added to autoclaved minimal medium). After 7 days, growth and spore production were scored.

Nickel uptake: Mycelium from 5 day old cultures grown in liquid minimal media was centrifuged, washed 2 times with aqua dest. and resuspended in polyvidon buffer (100 ml 50 mM K_2HPO_4/KH_2PO_4-buffer, pH 7.2, with 15.4 mg dithioerythrit and 2 g polyvinylpyrrolidon). Cells were disrupted using a French press (French Pressure Cell Press, SLM Instruments) and cytosol was separated from cell wall and membrane fragments by ultracentrifugation (Beckman LE-70 Ultracentrifuge, 140,000 g, 90 min). For chromatography, 1 ml cytosol was used (column XK 26/100, Amersham Biosciences with sephadex G50 fine). Protein was quantified by UV (Uvicord SII, Amersham Biosciences), nickel by ICP-MS. Fractions were eluted with aqua dest. and 5 ml fractions were collected (Frac 100, Amersham Biosciences). As size standards, BSA (66 kDa), ovalbumine (44.3 kDa) and α-chymotrypsinogene A (25.7 kDa) were used.

SOD enzyme activity: For native gel electrophoresis and activity staining, cytosol was concentrated (1:10) by lyophilization (Alpha 1–4, Christ GmbH, Osterode/Harz). After resuspension in polyvidon buffer, protein was detected by Bradford reagent (Brilliant Blue R Concentrate, Sigma; Bradford, 1976). Native PAGE was performed using 10% acryl amide gels. 50–60 µg protein were loaded per lane. Electrophoresis was carried out at 4 °C with 100 to 160 V. In-gel activity staining was performed as described (Beauchamp and Fridovich 1971). For inhibition assays, gel slices were incubated for 30 min (either with 5 mM KCN, 5 mM H_2O_2 or 50 mM phosphate buffer) before staining. 5 mM H_2O_2 and 5 mM KCN, respectively, were used for inhibition assays; $CuSO_4$ plus $ZnCl_2$, or $CrCl_3$, or $NiCl_2$ were used to test for activities in different heavy metal containing media. All tests were performed in triplicate.

Isolation and characterization of sodN: The gene *sodN* encoding NiSOD from *S. acidiscabies* E13 was identified by PCR using primers derived from the gene sequences of *S. coelicolor* and *S. seoulensis*, *sodN*-for: 5′-TGC CCC CAA GGT CAC GGT CAG-3′, *sodN*-rev: 5′-CAG GGC CTT CAG GGT GTC GTT-3′. For cloning and sequencing of *sodN*

the primer *sodN*-gesamt-for (5′-CCT AAG CCC (AT) (GC)G GGG GCA C-3′), derived from the *S. coelicolor* genome sequence was used with *sodN*-rev to amplify a fragment that starts at –59 nt. Since sequence homologies downstream of different *sodN* genes were lacking, a Touch down PCR with the primer *sodN*-gesamt-for was used. Touch down PCR products were controlled by Southern Blot analysis (Roche). The PCR products were cloned, electrotransferred (2,8 kV, 25 µF, 220 Ω, EC100 Electroporator, E-C Apparatus Corporation, Holbrook, USA) in competent *E. coli* DH5α and the fragments verified by restriction analyses before sequencing (JenaGen, Jena).

Results

Isolation of actinobacteria and selection of a multiple heavy metal resistant strain

Actinobacteria from nickel-rich environments and from uncontaminated soils were isolated. The soil samples were taken from a city park within the city of Jena as presumably uncontaminated site, and two locations with elevated nickel concentrations: geogenically nickel containing serpentine soil from Tuscany, Italy, and an anthropogenically contaminated site at the former uranium mining site Ronneburg in Thuringia, Germany. There, several other heavy metals are present at elevated concentrations together with the higher nickel contents of the soil. The nickel content of control soil was 4.5 ppb (translating to a µM range), of serpentine soil 87 ppb (less than 2 mM Ni) while the soil samples at the former uranium mining site contained 322 ppb Ni, 5 ppb Cd, 100 ppb Cu, 443 ppb Zn, 44 ppb Co and 2 ppb As (for Ni more than 5 mM).

From each site, strains were isolated and tested for growth on 5 mM nickel containing minimal media. Minimal media have to be used for such analyses in order to prevent complexation of the heavy metals with media components which would lead to over-estimation of resistance levels of the tested strains. The amount of sensitive strains not capable of growing with 0.2 mM Ni is clearly related to the origin of the soil samples, with 27% sensitive strains (10 out of 37) from control soil while from that soil only two resistant strains (5.4%) were recovered. Among the isolates from the other two sites, no strictly sensitive strains were recovered. From the serpentinite soil of Tuscany 18% (6 out of 33 strains) of the strains were resistant to more than 5 mM Ni, while that proportion at Wismut sites with highest contamination amounted to 35% (7 out of 20 strains). All together, the amount of resistant strains tolerating more than 0.2 mM nickel is gernerally high, while resistant bacteria capable of growing on minimal media with more than 10 mM $NiCl_2$ are exceptionally rare at all tested sites.

Table 1. Heavy metal resistances of actinobacterial isolates.

	Cu	Cd	Ni	Cr	Zn	Fe	Mn
S. acidiscabies E13	1.5	1.5	10	2.5	50	25	25
strain JE7	0	0	20	0	50	<10	5
strain JE12	0.5	0	20	1	25	<10	5

Three strains with high resistance towards nickel were tested in detail for different heavy metal tolerances. Table 1 shows that one isolate from Wismut soil classified as *S. acidiscabies* by 16S rDNA comparisons (Amoroso *et al.* 2000) exhibited multiple heavy metal resistances and in comparison showed the broadest range of resistance. While the two strains JE7 and JE12 are resistant to nickel and zinc only, *S. acidiscabies* E13 also tolerated copper, cadmium, chromium and iron. For the purpose of testing inifluence of heavy metals on expression of superoxide dismutases, this broad heavy metal resistance seemed ideally suited.

Since the influence of intracellular heavy metal was to be tested on superoxide dismutases, strain *S. acidiscabies* E13 was investigated for intracellular nickel accumulation. To test for nickel uptake, cells were grown in nickel containing minimal media. After incubation, supernatant nickel concentrations show slighly higher adsorption/uptake of *S. acidiscabies* E13 as compared to *S. coelicolor* (Tab. 2). Chromatography could then indicate that nickel was taken up into the cytosol (Fig. 1). The main fraction of cytosolic nickel eluted late with almost no protein present in the samples. Since high phosphorous contents are prevalent in these samples, we speculated that nickel might be preferentially bound to phosphate in cells of *S. acidiscabies* E13. Since this strain exhibited multiple heavy metal resistances and could take up heavy metals into the cell, superoxide dismutases were analyzed from this strain.

Table 2. Nickel retention from the medium in independent experiments.

	parallel 1	parallel 2	parallel 3	parallel 4	mean value	deviation
S. coelicolor	743	673	448	361	556	181
S. acidiscabies E13	1174	669	812	838	873	214

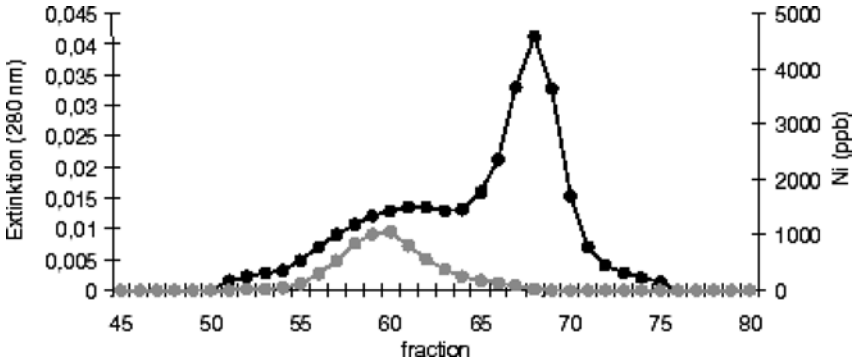

Figure 1. Size exclusion column chromatography of cytosol. Fractions were measured for nickel (grey graph) and protein content (dark graph).

Characterization of SODs from *S. acidiscabies* E13

Gel electrophoresis and activity staining could show one each of a nickel and an iron containing superoxide dismutase (Fig. 2) as shown by differential enzymatic repression studies. The identity of the nickel induced enzyme as NiSOD was verified by differential repression with hydrogen peroxide while KCN incubated enzyme showed approximatly 50% remaining activity.

Expression of both superoxide dismutases in media with different heavy metals was screened and the resulting superoxide dismutase activities tested by native gel electrophoresis and activity staining. While FeZn-SOD was repressed by nickel (Fig. 2A), chromium and combined copper/zinc and manganese showed slight effect on expression of *sodF* (Fig. 2B). Expression of *sodN* was enhanced by nickel (Fig. 2A, B). Although no nickel was added to the media containing the other heavy metals, NiSOD activity was visible while a culture without Ni or other heavy metals showed only a weak band for NiSOD (see Fig. 2A). Enzyme activity is already visible at very low nickel concentrations (10 μM), even in minimal media without supplementation of other heavy metals. Thus, the response on heavy metal stress seems to rely rather on NiSOD induction while FeZn-SOD expression is unchanged or even repressed under heavy metal stress. Therefore, the gene encoding NiSOD was analyzed.

Characterization of the gene encoding nickel-containing superoxide dismutase

Using primers derived from *sodN* of *S. coelicolor* and *S. seoulensis*, *sodN* of *S. acidiscabies* E13 was amplified and sequenced. The genomic sequence shows 92.9% nucleotide identity to that of *S. coelicolor* and 94.7% amino acid identity (Fig. 3). The gene consists of 396 bp which is translated to 131 amino acids. The conserved residues that contribute to nickel binding are homologous to known proteins (Youn *et al.* 1996b). The observed nucleotide divergencies are mostly mute, and only in 17 cases amino acid differences result from the different nucleotide sequences. Of these, only at three sites lead nucleotide exchanges to new amino acids which are not represented in either of the other four known sequences: T55V, T106/K106A, and K130Q. While position 106 seems variable with either T, K or A, the two remaining positions T55V and K130Q are new in the sequence of *S. acidiscabies* E13.

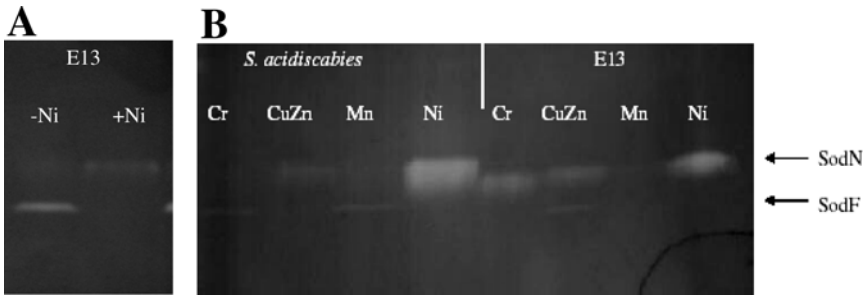

Figure 2. Expression of SODs under heavy metal inducing conditions. *S. acidiscabies* type strain and *S. acidscabies* E13 were compared after growth in heavy metal containing media.

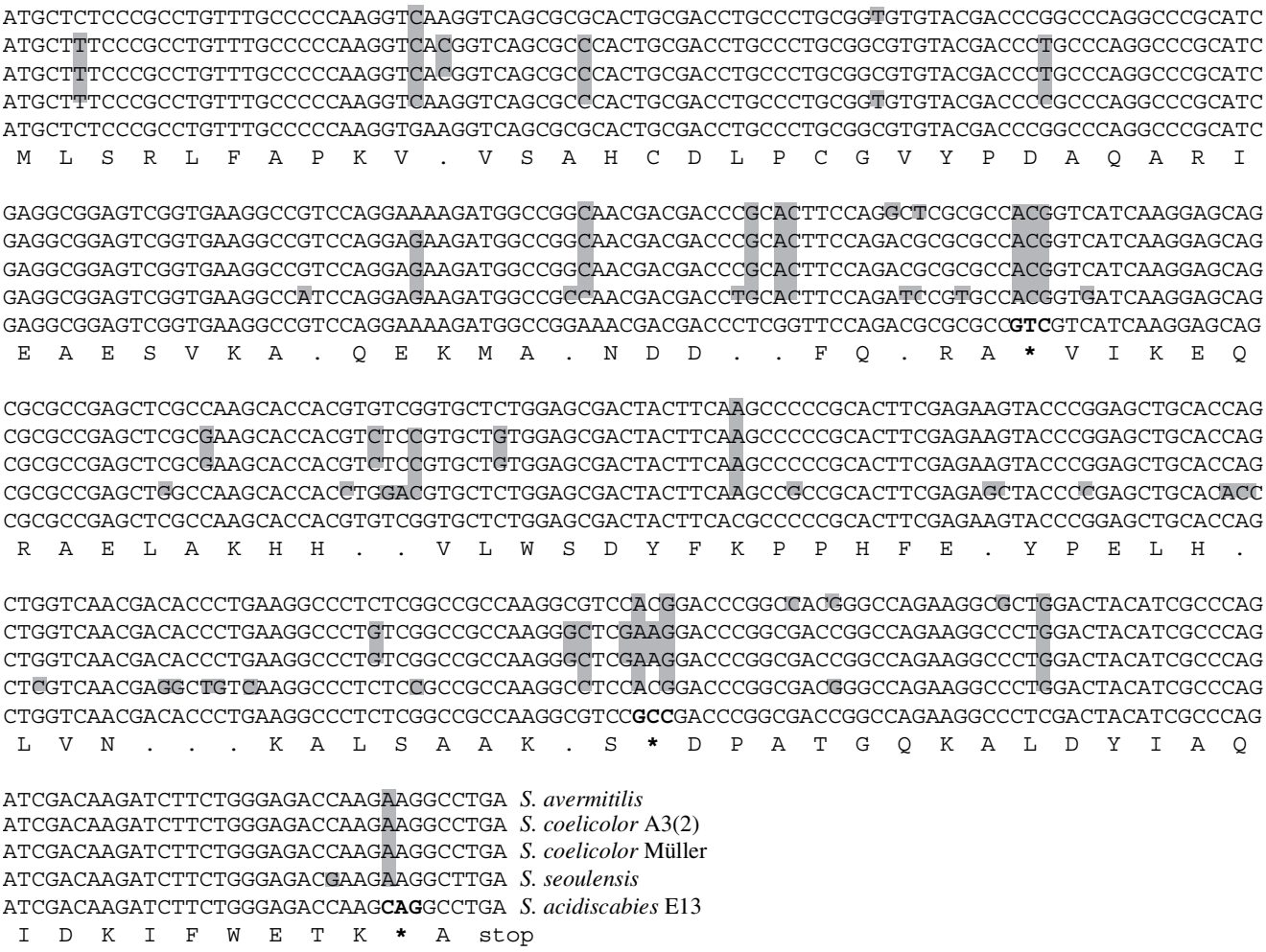

Figure 3. Alignment of the nucleotide sequences for *sodN* from *S. avermitilis*, *S. coelicolor* A3(2), *S. coelicolor* Müller, *S. seoulensis* and *S. acidiscabies* E13. Deviations in sequence are highlighted in grey. The resulting amino acid sequence is given below with variable positions indicated (.) and the three seites leading to new positions additionally shown (*) in bold.

Discussion

Evolution of heavy metal tolerance at the population level in geogenically versus anthropogenically contaminated soils

We could show that at both contaminated sites no sensitive strains were found while in an uncontaminated environment approximatly 25% of isolates were sensitive towards nickel. In addition, the long-term adaptation on serpentine soils naturally containing elevated nickel concentrations in the range of 1 to 2 mM led to significantly higher numbers (18%) of resistant strains tolerating 5 mM Ni. Still higher proportions of resistant strains were found at the Wismut site with 7 out of 20 tested strains adding up to 35% resistants. This correlates to the findings of higher proportions of nickel resistant strains in different environments with elevated nickel contents like serpentine and serpentinite soils, or the rhizophere of nickel hyperaccumulating plants (Mengoni *et al.* 2001, 2004, Pal *et al.* 2005, Roane and Kellogg 1996, Shallari *et al.* 1998).

The survey also could show that even in presumably non-contaminated environments, some strains can be found which are resistant. This finding examplifies the possibility of natural attenuation which, on polluted soils, is dependent on resident and airborn deposition of resistant strains to initiate a resistant microflora and restore soil microbiology (Aleem *et al.* 2003, Delorme *et al.* 2001, Francisco *et al.* 2002, Hery *et al.* 2003, Rajapaksha *et al.* 2004).

The long-term adaptation to nickel rich environments has not yielded strains which have assembled a higher resistance and especially not the co-resistance towards other metals that would allow specific incor-

poration of, e.g., cobalt. Such a mechanism might have been expected since from Gram-negative bacteria nickel resistance mechanisms are known which at the same time permit growth on nickel and cobalt, or on cobalt, chromium and zinc containing media (Grass *et al.* 2000, Grosse *et al.* 2004). The survey clearly shows that not a single resistance gene is to be expected that covers all different metal resistances, but that multiple resistance factors are co-assembled in multiresistant strains like *S. acidiscabies* E13. Two strains from the control soil, although exhibiting high nickel resistance, were not tolerant towards other heavy metals thus most likely featuring a nickel specific resistance factor.

Regulation of SOD gene expression

S. acidiscabies E13 produced NiSOD from the gene sequenced and one isozyme of FeSOD. As described for *S. seoulensis* and *S. coelicolor*, Ni inhibited FeSOD expression and strongly induced NiSOD expression, probably because of higher turnover numbers of the Ni containing enzyme (Kim *et al.* 1998a, b). Activity staining of native gels could show that heavy metals other than nickel are able to induce *sodN* expression and from these gels it is seen that at the same time these other metals (with the exception of Mn which also stimulates growth) reduce *sodF* expression.

The gene encoding NiSOD could be identified using a PCR approach with primers derived from known *sodN* genes derived from the databases. The gene in *S. acidiscabies* E13 proved to be homologous to the known genes from other species of the genus *Streptomyces* with high sequence identity (Choudhury *et al.* 1999, Kim *et al.* 1998a, b, Lee *et al.* 2002).

The transcriptional regulation of FeSODs is prone to be mediated by a Ni sensing factors. For *S. coelicolor* A3(2) *sodF1* repression by Ni, a *cis*-acting region (–16 to +30) could be identified (Chung *et al.* 1999). For *S. griseus sodF,* a putative regulatory sequence was also defined and two genes downstream of *sodF*, *srnR* and *srnQ* were detected which are thought to perform the negative regulation (Kim *et al.* 2000, 2003). However, we could not detect homologous sequences to *srnRQ* in the genomes of *S. avermitilis* or *S. coelicolor*.

S. acidiscabies E13 *sodN* role in heavy metal response

The screening for heavy metal resistant strains has proved successful and provided a strain that can be grown under elevated heavy metal concentrations retaining NiSOD activity. This is related to the multifactorial resistance mechanisms and implies NiSOD to be one of the heavy metal resistance mediating proteins, especially since heavy metal stress is known to enhance superoxide formation. Different heavy metals induced the expression which allows further studies using overexpression strains from genetic transformation. Thus, this strain can be used to further characterize NiSOD in heavy metal stress. It also will enable us to investigate the enzyme biochemically, since the crystal structure of the enzyme has now been published (Barondeau *et al.* 2004, Szilagyi *et al.* 2004, Würges *et al.* 2004). At the same time, gene regulation can be studied in detail using the data assembled from this first screening of strains for NiSOD investigation.

Acknowledgements

We would like to thank L. Zeggel, D. Merten, M. Carlsohn, A. Rassmann, J. Nüske, and P. Mitscherlich for help. This work was financed through SFB 436 by the DFG.

References

Aleem, A., Isar, J. and Malik, A., 2003. Impact of long-term application of industrial wastewater on the emergence of resistance strains in *Azotobacter chroococcum* isolated from rhizosphere soil. Bioresour. Technol., **86**, 7–13.

Amoroso, M.-J., Schubert, D., Mitscherlich, P., Schumann, P. and Kothe, E., 2000. Evidence for high affinity nickel transporter genes in heavy metal resistant *Streptomyces* spec. J. Basic Microbiol., **40**, 295–201.

Barondeau, D.P., Kassmann, C.J., Bruns, C.K., Tainer, J.A. and Getzoff, E.D., 2004. Nickel superoxide dismutase structure and mechanism. Biochemistry, **43**, 8038–8047.

Beauchamp, C. and Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem., **44**, 276–287.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**, 248–254.

Choudhury, S.B., Lee, J.W., Davidson, G., Yim, Y.I., Bose, K., Sharma, M.L., Kang, S.O., Cabelli, D.E. and Maroney, M.J., 1999. Examination of the nickel site structure and reaction mechanism in *Streptomyces seoulensis* superoxide dismutase. Biochemistry, **23**, 3744–3752.

Chung, H.J., Choi, J.H., Kim, E.J., Cho, Y.H. and Roe, J.H., 1999. Negative regulation of the gene for Fe-containing superoxide dismutase by an Ni-responsive factor in *Streptomyces coelicolor*. J. Bacteriol., **181**, 7381–7384.

Ciriolo, M.R., Civitareale, P., Carri, M.T., De Martino, A., Galiazzo, F. and Rotilio, G., 1994. Purification and characterization of Ag, Zn-superoxide dismutase from *Saccharomyces cerevisiae* exposed to silver. J. Biol. Chem., **14**, 25783–25787.

Culotta, V.C., Joh, H., Lin, S., Slekar, K.H. and Strain, J., 1995. A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. J. Biol. Chem., **270**, 29991–29997.

Delorme, T.A., Gagliardi, J.V., Angle, J.S. and Chaney, R.L., 2001. Influence of the zinc hyperaccumulator *Thlapsi caerulescens* J. and C. Presl. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial populations. Can. J. Microbiol., **47**, 773–776.

Francisco, R., Alpoim, M.C. and Morais, P.V., 2002. Diversity of chromium-resistant and -reducing bacteria in a chromium-contaminated activated sludge. Appl. Microbiol., **92**, 837–943.

Fridovich, I., 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem., **64**, 97–112.

Geslin, C., Llanos, J., Prieur, D. and Jeanthon, C., 2001. The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. Res. Microbiol., **152**, 901–905.

Grass, G., Grosse, C. and Nies, D.H., 2000. Regulation of the *cnr* cobalt and nickel resistance determinant from *Ralstonia* sp. strain CH34. J. Bacteriol., **182**, 1390–1398.

Grosse, C., Anton, A., Hoffmann, T., Franke, S., Schleuder, G. and Nies, D.H., 2004. Identification of a regulatory pathway that controls the heavy-metal resistance system Czc *via* promotor *czcNp* in *Ralstonia metallidurans*. Arch. Microbiol., **182**, 109–118.

Hery, M., Nazaret, S., Jaffre, Z., Normand, P. and Navarro, E., 2003. Adaptation to nickel spiking of bacterial communities in neocaledonian soil. Environm. Microbiol., **5**, 3–12.

Kim, E.J., Chung, H.J., Suh, B., Hah, Y.C. and Roe, J.H., 1998a. Expression and regulation of the *sodF* gene encoding iron and zinc-containing superoxide dismutase in *Streptomyces coelicolor* Müller. J. Bact., **180**, 2014–2020.

Kim, E.J., Chung, H.J., Suh, B., Hah, Y.C. and Roe, J.H., 1998b. Transcriptional and post-transcriptional regulation by nickel of *sodN* gene encoding nickel-containing superoxide dismutase from *Streptomyces coelicolor* Müller. Mol. Microbiol., **27**, 187–195.

Kim, J.S., Jang, J.H., Lee, J.W., Kang, S.O., Kim, K.S. and Lee, J.K., 2000. Identification of cis site involved in nickel responsive transcriptional repression of *sodF* gene coding for Fe- and Zn-containing superoxide dismutase of *Streptomyces griseus*. Biochim. Biophys. Acta, **1493**, 200–207.

Kim, J.S., Kang, S.O. and Lee, J.K., 2003. The protein complex composed of nickel-binding SrnQ and DNA binding motif-bearing SrnR of *Streptomyces griseus* represses *sodF* transcription in the presence of nickel. J. Biol. Chem., **278**, 18455–18463.

Leclere, V., Boiron, P. and Blondeau, R., 1999. News and notes: diversity of superoxide-dismutases among clinical and soil isolates of *Streptomyces* species. Curr. Microbiol., **39**, 365–368.

Lee, J.W., Roe, J.H. and Kang, S.O., 2002. Nickel-containing superoxide dismutase. Methods Enzymol., **349**, 90–101.

Martin, M., Byers, B.R., Olson, M.O.J., Salin, M.L., Arceneaux, J.E.L. and Tolbert, C., 1986. A *Streptococcus mutans* superoxide dimutase that is active with either manganese or iron as cofactor. J. Biol. Chem., **261**, 9361–9367.

Meier, B., Sehn, A.P., Sette, M., Paci, M., Desideri, A. and Rotilio, G., 1994. *In vivo* incorporation of cobalt into *Propionibacterium shermanii* superoxide dismutase. FEBS Lett., **348**, 283–286.

Mengoni, A., Barzanti, R., Gonnelli, C., Gabbrielli, R. and Bazzicalupo, M., 2001. Characterization of nickel-resistant bacteria isolated from serpentine soil. Environ. Microbiol., **3**, 691–698.

Mengoni, A., Grassi, E., Barzanti, R., Biondi, E.G., Gonnelli, C., Kim, C.K. and Bazzicalupo, M., 2004. Genetic diversity of bacterial communities of serpentine soil and of rhizosphere of the nickel-hyperaccumulator plant *Alyssum bertolonii*. Microb. Ecol., **48**, 209–217.

Pal, A., Dutta, S., Mukherjee, P.K. and Paul, A.K., 2005. Occurence of heavy metal-resistance in microflora from serpentine soil of Andaman. J. Basic Microbiol., **45**, 207–218.

Palenik, B., Brahamsha, B., Larimer, F.W., Land, M., Hauser, L., Chain, P., Lamerdin, J., Regala, W., Allen, E.E., McCarren, J., Paulsen, I., Dufresne, A., Partensky, F., Webb, E.A. and Waterbury, J., 2003. The genome of a motile marine *Synechococcus.* Nature, **424**, 1037–1042.

Rajapaksha, R.M., Tobor-Kaplon, M.A. and Baath, E., 2004. Metal toxicity affects fungal and bacterial activities in soil differently. Appl. Environ. Microbiol., **70**, 2966–2973.

Ray, G. and Husain, S.A., 2002. Oxidants, antioxidants and carcinogenesis. Indian J. Exp. Biol., **40**, 1213–1232.

Roane, T.M. and Kellogg, S.T., 1996. Characterization of bacterial communities in heavy metal contaminated soils. Can. J. Microbiol., **42**, 593–603.

Shallari, S., Schwartz, C., Hasko, A. and Morel, J.L., 1998. Heavy metals in soils and plants of serpentine and industrial sites of Albania. Sci. Total Environ., **209**, 133–142.

Stohs, S.J. and Bagchi, D., 1993. Oxidative mechanisms in the toxicity of metal ion. Free Radical Biol. Med., **18**, 321–336.

Szilagyi, R.K., Bryngelson, P.A., Maroney, M.J., Hedman, B., Hodgson, K.O. and Solomon, E.I., 2004. S K-edge X-ray absorption spectroscopic investigation of the Ni-containing superoxide dismutase active site: new structural insight into the mechanism. J. Am. Chem. Soc., **126**, 3018–3019.

Würges, J., Lee, J., Yim, Y., Yim, H., Kang, S. and Carugo, K.D., 2004. Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. Proc. Natl. Acad. Sci. USA, **101**, 8569–8574.

Youn, H.-D., Kim, E.-J., Roe, J.-H., Hah, Y.C. and Kang, S.-O., 1996a. A novel nickel-containing superoxide dismutase from *Streptomyces* spp. Biochem. J., **318**, 889–896.

Youn, H.-D., Youn, H., Lee, J.-W., Yim, Y.-I., Lee, J.K., Hah, Y.C. and Kang, S.-O., 1996b. Unique isoenzymes of superoxide dismutase in *Streptomyces griseus.* Arch. Biochem. Biophys., **334**, 341–348.

Zhang, Z., Wang, Y. and Ruan, J., 1997. A proposal to revive the genus *Kitasatospora* (Omura, Takahashi, Iwai, and Tanaka 1982). Int. J. Syst. Bacteriol., **47**, 1048–1054.

3.2 Heavy metal resistance in actinobacteria of the former uranium mining area near Ronneburg

André Schmidt, Götz Haferburg, Astrid Schmidt and Erika Kothe

In Proceedings des Internationalen Bergbausymposiums WISMUT 2007, Stilllegung und Revitalisierung von Bergbaustandorten zur nachhaltigen Regionalentwicklung, Wismut GmbH, 2007.

HEAVY METAL RESISTANCE IN ACTINOBACTERIA OF THE FORMER URANIUM MINING AREA NEAR RONNEBURG

André Schmidt, Götz Haferburg, Astrid Schmidt, Erika Kothe[1]

Actinobacteria including streptomycetes are grampositive bacteria with a high GC-content. The group is characterized by a complex life cycle including mycelial growth and spore production. Streptomycetes are prominent producers of secondary metabolites, among them a large number of antibiotics. Although they represent a wide-spread soil colonizing group little is known about their ability to exist in strongly contaminated soils. Low concentrations of heavy metals are not harmful to microorganisms, some metals like nickel, cobalt or zinc are even essential for the synthesis and function of enzymes, cofactors or transcriptional regulators. However, high concentrations of heavy metals in the environment lead to an increasing intracellular concentration with the consequence of inhibition of enzymes or DNA damage. Therefore, microorganisms in heavy metal contaminated soils are in need of special resistance mechanisms to cope with this environment. The banks of the Gessenbach creek are characterized by exceedingly high concentrations of heavy metals and radionuclides as a consequence of the permanent inflow of acid mine drainage water over several decades of mining activity. Therefore, the creek appears to be an interesting location for the search for heavy metal resistant microorganisms.

Isolation of nickel-resistant actinobacteria

Soil samples were collected from the bank of Gessenbach in a 900 m long section starting after the point where it merges with the Badergraben. The samples were analyzed for nickel resistant actinobacterial populations, microbial activity, heavy metal content and pH.

Comparison with non-contaminated control soils revealed that most heavy metals, including uranium, occur at elevated concentrations. At the polluted sites, soil pH was about 4.5 and microbial activity tested *via* soil respiration was significantly reduced. However, from most sample sites nickel resistant actinobacteria could be isolated. Further experiments revealed that several strains also are resistant to high concentration of cadmium, cobalt and zinc.

Molecular analysis of heavy metal resistance

Heavy metal resistance mechanisms with the exception of mercury have not yet been identified for actinobacteria on a molecular level. In chromatography experiments it could be shown that *Streptomyces acidiscabies* E13 is able to store high amounts of nickel in the cytosol. Additionally, it could be shown via pulse field gel electrophoresis that this strain contains a plasmid on which resistance factors like efflux transporters could be encoded. Another strain, *Streptomyces tendae* F4, is able to grow on high concentrations of cadmium. Plating experiments revealed that it excretes a chelating substance that lowers the bioavailability of cadmium and thus enables adjacent cadmium sensitive strains to grow (Fig. 1).

[1] Friedrich-Schiller-University, Institute of Microbiology – Microbial Phytopathology, Neugasse 25, 07745 Jena

Resistance mechanisms of the strains found on the banks of Gessenbach will be analyzed in detail. First results were obtained by the application of two dimensional protein gel electrophoresis followed by mass spectrometry that allows comparing the protein expression of a bacterial strain under different cultivation conditions. For the nickel resistant strain *Streptomyces acidiscabies* E13 proteins could be identified that were induced under the influence of nickel.

In addition, we will analyze the role of plasmids found in highly resistant strains and whether these plasmids can be transferred to other strains.

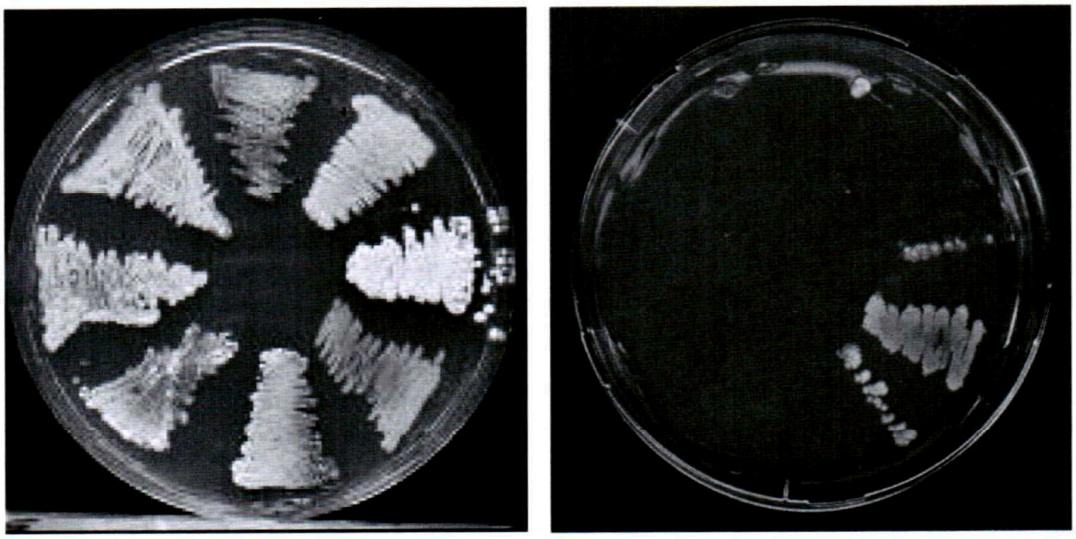

Fig.1. Comparison of actinobacterial growth on minimal medium (left) and on cadmium amended medium (right). Cadmium resistant strain Streptomyces tendae F4 enables adjacent cadmium sensitive strains to grow.

References

Nies, D. H. (2003): Efflux-mediated heavy metal resistance in prokaryotes, FEMS Microbiol Rev 27, 313-339

Ravel, J., Schrempf, H., Hill, R. T. (1998): Mercury resistance is encoded by transferable giant linear plasmids in two Chesapeake Bay Streptomyces strains. Appl. Environ. Microbiol., 64, 3383-3388

Schmidt, A., Haferburg, G., Sineriz, M., Merten D., Büchel, G., Kothe, E. (2005): Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils, Geochem., 65, 131-144

Schmidt, A., Schmidt, A., Haferburg G., Kothe, E. (2007): Superoxide dismutases of heavy metal resistant streptomycetes. J. Basic Microbiol., 47, 65-62

3.3 Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area

André Schmidt, Götz Haferburg, Astrid Schmidt, Ulrike Lischke, Dirk Merten, Felicia Gherghel, Georg Büchel and Erika Kothe

Chemie der Erde (2009) 69, S2, 35–44

Available online at www.sciencedirect.com
ScienceDirect
Chemie der Erde 69 (2009) S2, 35–44
CHEMIE der ERDE GEOCHEMISTRY
www.elsevier.de/chemer

Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area

André Schmidt[a,*], Götz Haferburg[a], Astrid Schmidt[a], Ulrike Lischke[a], Dirk Merten[b], Felicia Ghergel[a], Georg Büchel[b], Erika Kothe[a]

[a]*Biological-Pharmaceutical Faculty, Institute of Microbiology, Friedrich-Schiller-University, Neugasse 25, 07743 Jena, Germany*
[b]*Faculty of Chemistry and Earth Sciences, Institute of Earth Sciences, Friedrich-Schiller-University, Burgweg 11, 07749 Jena, Germany*

Received 8 August 2007; accepted 8 November 2007

Abstract

Contamination of large areas by industrial or mining activities is a serious environmental problem. Selection pressure in these polluted habitats led to adaptation in microorganisms now containing special resistance mechanisms as a result to the permanent exposure to exceedingly high concentrations of heavy metals. Banks of a creek in a former uranium mining area near Ronneburg, Germany, were analyzed with regard to highly heavy metal resistant actinobacterial strains, with special attention to nickel, which is both harmful contaminant and essential for incorporation in enzymes. In this work, seven *Streptomyces* strains that show a high resistance to nickel are presented. Two *Streptomyces mirabilis* strains are able to grow on concentrations higher than 100 mmol/l nickel. Additional experiments revealed that both strains are also able to cope with 100 mmol/l zinc. Both strains are differing in plasmid equipment and production of secondary metabolites on nickel-amended complex medium. The survival at the contaminated site could be mimicked using media prepared with soil that present the composition of mobile and adsorbed heavy metals in axenic culture that are present in the environment and determine the soil microflora composition there.

Keywords: Heavy metal resistance; Nickel; Bioavailability; *Streptomyces*

1. Introduction

Streptomycetes are gram-positive bacteria with a high guanine–cytosine content. They belong to the group of actinobacteria and are characterized by a complex life cycle and the production of an amount of secondary metabolites which often find a use in medicinal applications (Hopwood, 2006).

Although they represent a widespread soil-colonizing group (Kieser et al., 2000), little is known about their presence in heavy metal contaminated soils. Heavy metals in low concentrations are not harmful to microorganisms, elements like nickel or zinc are even essential because of their incorporation in enzymes or cofactors (Watt and Ludden, 1999). Nevertheless, high concentrations of heavy metals in the environment lead to an increasing intracellular concentration with the

*Corresponding author. Tel.: +49 3641 949391; fax: +49 3641 949292.
E-mail address: smiddy74@yahoo.de (A. Schmidt).

0009-2819/$ - see front matter © 2007 Elsevier GmbH. All rights reserved.
doi:10.1016/j.chemer.2007.11.002

consequence of inhibition of enzymes or DNA damage by the production of reactive oxygen species or irreversible binding to the active centers of enzymes (Lopez-Maury et al., 2002). Abbas and Edwards (1989) have shown that growth and synthesis of antibiotics and pigments of *Streptomyces* strains were negatively affected by the presence of different heavy metals. Comparable results were obtained by Raytapadar et al. (1995) for *Streptomyces galbus*, whereas Smylla and Mroczkowska-Badner (1991) showed that increasing concentrations of cadmium lead to inhibition of growth of all investigated *Streptomyces* strains. To avoid these consequences, several bacteria possess resistance mechanisms like superoxide dismutases (Kim et al., 2003), efflux transporters (Anton et al., 1999; Mergeay et al., 2003) or metallothioneins (Olafson et al., 1988; Robinson et al., 2001). A prominent heavy metal resistant strain with an effectively working efflux transporter system is the gram-negative proteobacterium *Cupriavidus metallidurans* CH34 (Mergeay et al., 1985; Nies et al., 1987). This organism contains two megaplasmids, pMOL28 and pMOL30, on which the resistance determinants *czc* and *cnr* are encoded. The corresponding transporters consist of three structural subunits and provide in the case of *czc* resistance against cobalt, zinc and cadmium, and in the case of *cnr* resistance towards cobalt and nickel.

For the genus *Streptomyces*, plasmids have been described that encode heavy metal resistance against mercury (Ravel et al., 1998).

Soils that contain a high content of heavy metals like serpentine soil (Idris et al., 2004, 2006) are natural habitats of communities with high levels of tolerance towards the prominent metals (Baath et al., 1998). In addition to these natural environments, strongly contaminated areas often are a result of industrial activity or mining (Shevchenko et al., 2003). A former uranium mining area exploited from 1952 to 1990 in Eastern Thuringia, Germany, is one of these anthropogenically contaminated environments (Merten et al., 2004). The banks of creeks in this area are characterized by exceedingly high concentrations of heavy metals as a consequence of the permanent inflow of acid mine drainage water over several decades (Geletneky et al., 2002) and thus appear to be interesting sites for the search for heavy metal resistant *Streptomyces* strains.

The strains presented in this paper were analyzed with regard to their resistance towards a range of heavy metals and their ability to grow on agar amended with a contaminated soil. Special attention was paid to nickel which usually occurs as a co-contaminant of uranium and further is part of the active center of a nickel-containing superoxide dismutase found in *Streptomyces* (Youn et al., 1996). This dual role of nickel as essential as well as toxic element necessitates specific measures for ensuring an intracellular homeostasis, which in turn might have led to the evolution of specific resistance factors. In this paper, we describe new *Streptomyces* isolates highly resistant to nickel and try to predict the different molecular strategies that the strains employ to gain nickel resistance.

2. Materials and methods

2.1. Isolation and growth of nickel-resistant *Streptomyces* strains

Soil sample collection was undertaken according to Schmidt et al. (2005) at points P1 through P18 and K7 (Schmidt et al., 2005). Isolation was performed with soil dried at room temperature. Because of the fact that spore production is strongly inhibited under the influence of heavy metals (Schmidt et al., 2005), the soil was not heated to 80 °C like in classical procedures for the isolation of streptomycetes from soil samples (Amoroso et al., 2000). Fungal growth could not be detected on minimal medium agar plates containing 15 mmol/l $NiCl_2$. Five hundred milligrams soil was resuspended in 1.5 ml distilled water and shaken for 30 min. One hundred microliters of the suspension was plated on minimal medium (0.5 g/l L-asparagine, 0.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4$, 0.01 g/l $FeSO_4$, 10 g/l glucose, pH 6.92 after autoclaving) agar plates containing 15 mmol/l $NiCl_2$. After 10 days of growth, different actinobacterial morphotypes were tested on 30 and 50 mmol/l $NiCl_2$. The strains with the highest resistance towards $NiCl_2$ were used for further experiments.

Mycelium for DNA extraction was taken from a minimal medium agar plate after 1 week of growth at 28 °C and transferred into an eppendorf tube. After the addition of 50 µl distilled water, the mycelium was frozen in liquid nitrogen, cracked with a pestle and then heated for 1 min at 50 °C. This procedure beginning with the freezing was repeated five times and then the sample was heated at 99 °C for 15 min and centrifuged for 2 min at 13,000 rpm. The supernatant was used for polymerase chain reaction (PCR).

Determination of heavy metal resistance was performed with minimal medium agar plates to avoid the complexation of heavy metals with ingredients of the medium. Stock solutions ($NiCl_2$, $NiSO_4$, $CoCl_2$, $CdCl_2$, $AlCl_3$, $CuSO_4$, $ZnSO_4$) were sterile filtered and added after autoclaving. Growth was evaluated after 10 days of cultivation at 28 °C.

Streptomyces coelicolor A3(2) was used as a control strain sensitive against rising concentrations of heavy metals (Schmidt et al., 2005).

Following heavy metal concentrations were analyzed: $NiCl_2$: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150 mmol/l; $NiSO_4$: 100, 110, 120, 130, 140, 150 mmol/l; $CoCl_2$: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mmol/l; $CdCl_2$: 0.5, 1, 1.5, 2, 2.5 mmol/l; $AlCl_3$: 0.5, 1, 2, 3, 4 mmol/l; $CuSO_4$: 1, 2, 3, 4, 5 mmol/l; $ZnSO_4$: 20, 40, 60, 80, 100 mmol/l.

To compare the effect of media for growth on high nickel concentrations, tryptic soy broth (TSB) medium agar plates were additionally used as representative for a complex medium. The three strains with the highest resistance towards nickel on nickel-amended minimal medium, *Streptomyces mirabilis* P16B-1, *S. mirabilis* K7A-1 and *S. mirabilis* P10A-3, were tested.

Growth and pigment production were evaluated after 20 days of growth at 28 °C using following concentrations of $NiCl_2$: 20, 40, 60, 80, 100 mmol/l.

2.2. Soil agar and soil extract agar

Soil is much more complex than a well-defined medium and contains a range of organic and inorganic substances. The soils of the banks of the creek Gessenbach contain a range of heavy metals and radionuclides. Thus, we employed plating on soil and soil extract agar in an approach to study bio–geo interactions. It should reveal whether resistances against metals which were measured on amended medium agar plates correlate with the growth on agar plates amended with contaminated soil or its extract.

For the preparation of soil agar plates, soil of the strongly contaminated site K7 dried at room temperature was used. 12.5 g soil was added to 50 (1:5), 75 (1:7) and 100 ml (1:9) medium with the final concentrations of medium contents corresponding to minimal medium. After the addition of agar (16 g/l), the sample was autoclaved and poured into agar plates.

Soil extract was prepared from 100 g soil of soil sample K7 in close vicinity to P16 (Schmidt et al., 2005), dried at room temperature and suspended in 150 ml distilled water, decanted and filtered with a 0.22-μm filter. To 50 ml of the soil extract, ingredients of minimal medium and agar (16 g/l) were added in corresponding amounts to reach final concentrations of medium contents corresponding to minimal medium. The sample was autoclaved and poured into agar plates.

Growth on soil extract and soil agar was evaluated after 10 days.

2.3. Soil pH and sequential extraction

Soil was dried at room temperature and then sieved (<2 mm). To 10 g of the soil, 25 ml distilled water was added. The sample was thoroughly stirred. After incubation for 1 h, the pH of the supernatant was measured by pH meter (inoLab pH 720, WTW GmbH, Weilheim, Germany).

The total heavy metal content of a soil does not reveal the bioavailable amount of heavy metals. Using sequential extraction, different fractions are separated by the use of different extraction agents.

Sequential extraction was performed according to Zeien and Bruemmer (1989). The first two fractions, the mobile and the specifically adsorbed fraction, are considered the bioavailable fractions. The digestion for the measurement of the total content of elements in a soil sample was performed with a pressure digestion system (DAS, PicoTrace, Germany). One hundred milligrams of the soil sample (ground to <63 μm) was mixed with 4 ml 40% HF (Suprapur) and 4 ml 70% $HClO_4$ (Suprapur). The sample was heated up to 180 °C in closed TFM vessels for 12 h. After cooling down, the acids were evaporated at a temperature of 180 °C by the use of a special hood. The residue was brought into solution by adding 2 ml 65% HNO_3 (subboiled), 0.6 ml 30% HCl and 7 ml pure water at a temperature of 150 °C. Sample site P16 (Schmidt et al., 2005) was chosen because the most nickel-resistant strain *S. mirabilis* P16B-1 was found in this soil. A soil sample from the non-contaminated Jenzig area in Jena, Germany, was analyzed as a control.

2.4. Strain identification

For the identification of the isolated strains, primer pair 243f (5′-GGA TGA GCC CGC GGC CTA-3′) and A3r (5′-CCA GCC CCA CCT TCG AC-3′) was used to amplify 16S rDNA (Monciardini et al., 2002). PCR was performed (5 min 94 °C, 35 cycles of 30 s 94 °C, 30 s 58 °C and 30 s 72 °C, followed by 10 min of 72 °C) and PCR products were analyzed on agarose gels. Subsequently, DNA was extracted directly from the PCR tube with SureClean from Bioline (London, UK). Sequencing was performed by JenaBiosciences (Jena, Germany).

2.5. Pulse-field gel electrophoresis

Strains were precultured for 24 h in 50 ml YEME medium (340 g/l sucrose, 10 g/l glucose, 5 g/l bacto-peptone, 3 g/l yeast extract, 3 g/l malt extract, 2 ml 2.5 M $MgCl_2$ after autoclaving) at 28 °C. Five milliliters of this culture were diluted in 50 ml YEME medium with 0.5% glycine. After 24 h of growth, the mycelium was collected by centrifugation and the pellet was washed twice with SucTE buffer (25 mmol/l Tris/HCl, 25 mmol/l EDTA, 300 mmol/l sucrose, pH 8.0). After resuspension in 5 ml SucTE, an optical density between 1.8 and 2.2 was achieved by dilution. Four hundred microliters of the mycelium suspension were heated to 50 °C, mixed with 800 μl of a low melting agarose and then pipeted in a cooled blockformer (BioRad) on ice. Subsequently, the blocks were incubated in 5 ml SucTE with 7.9 mg lysozyme from chicken egg white (Fluka, 92717 U/mg) for 1 h at 37 °C, incubated in lysis buffer (0.5 M EDTA, 1% lauroyl sarcosinate, 0.1 mmol/l Tris/HCl, pH 9.5) with proteinase K (1 mg/ml) and incubated overnight at 50 °C. The blocks were washed twice with TE buffer (10 mmol/l Tris/HCl, 1 mmol/l EDTA, pH 8.0) and stored in 0.5 M EDTA, pH 9.5, until electrophoresis.

In preparation for the pulse-field gel electrophoresis (PFGE), the blocks were incubated overnight in electrophoresis buffer (0.5 × TBE buffer (1 × TBE: 89 mmol/l Tris, 89 mmol/l boric acid, 2 mmol/l EDTA, pH 8.0)). For electrophoresis, a 1% agarose gel (12.5 × 14 cm; 1 g Seakem Gold Agarose (Biozym, Germany) in 100 ml 0.5 × TBE buffer) was used. Before the deposition of the gel the gel chamber was washed with 0.5 × TBE buffer.

The blocks were sealed in the gel slots with 1% low melting agarose, and PFGE was performed (14 °C, pump position 60, angle 120°, 24 h at 150 V, pulse 25–90 s, ramped) using a size marker (Lambda Ladder, New England Biolabs, USA) for size determination.

The gel was stained with ethidium bromide and bands recorded under UV light.

3. Results

3.1. Identification of highly resistant streptomycetes

Twenty actinobacterial morphotypes that were able to grow on 15 mmol/l $NiCl_2$ were isolated, six of them also

showed growth up to 30 mmol/l $NiCl_2$, and six further were able to grow on 50 mmol/l $NiCl_2$. The six strains with the highest nickel resistance were further analyzed together with one strain showing a good growth on $NiCl_2$ concentrations up to 30 mmol/l.

16S rDNA analysis revealed that the chosen nickel-resistant isolates belong to the genus *Streptomyces*.

The three strains with the highest nickel resistance levels all belong to the species *S. mirabilis*. Furthermore, two isolates were identified as *Streptomyces chromofuscus*, one as *Streptomyces prunicolor* and one as *Streptomyces naganishii* (Table 1).

The isolated strains were tested for their resistance against increasing concentrations of $AlCl_3$, $CdCl_2$, $CoCl_2$, $CuSO_4$, $NiCl_2$ and $ZnSO_4$ in minimal medium (Table 1, Fig. 1). $NiSO_4$ was also tested for concentrations higher than 100 mmol/l to reveal whether this salt shows other toxicity (Table 1, Fig. 1). The results show that all isolates are able to grow on high concentrations of nickel. Two strains, *S. mirabilis* P10A-3 and *S. mirabilis* P16B-1 were able to grow on $NiSO_4$ concentrations higher than 100 mmol/l. *S. mirabilis* P10A-3 also shows the ability to grow on 100 mmol/l $NiCl_2$, but pigment production seems to be inhibited. The strains *S. mirabilis* P16B-1, *S. mirabilis* K7A-1 and *S. mirabilis* P10A-3 were able to grow on 100 mmol/l $ZnSO_4$, while strain P4B-1 shows the ability to cope with $CoCl_2$ concentrations of up to 10 mmol/l.

$AlCl_3$ and $CuSO_4$ were tolerated only at low concentrations up to 2 mmol/l. Nevertheless, all isolates showed a higher resistance to aluminum (1 mmol/l) than *S. coelicolor* A3(2) (0.5 mmol/l). Against cadmium all isolates were sensitive and none of the strains were able to grow on 0.5 mmol/l cadmium.

In summary, all isolates obtained from a metalliferous habitat were able to grow on higher concentrations of zinc, aluminum and nickel as compared to the control *S. coelicolor* A3(2), while for cadmium and copper, resistance could not be detected.

3.2. Capability to cope with the conditions in the contaminated environment

The total content of a heavy metal in a soil does not give any information on its bioavailability. Therefore, we used sequential extraction to obtain a data set on

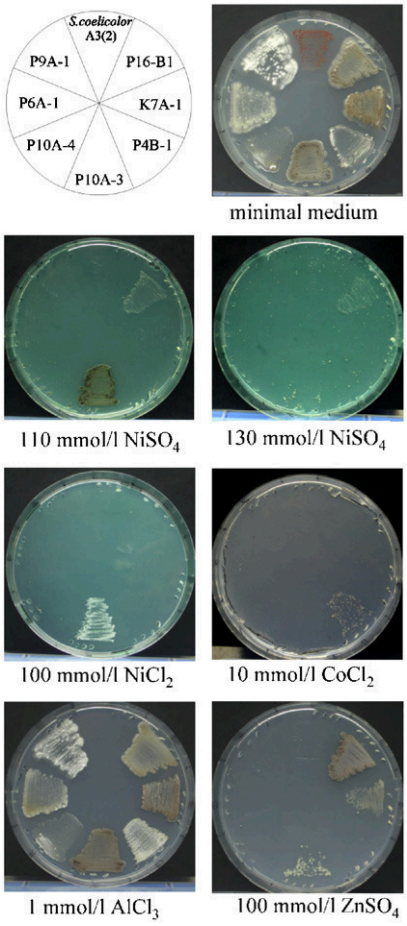

Fig. 1. Growth of highly nickel-resistant isolates on different heavy metals and aluminum. Each plate has a diameter of 87 mm.

Table 1. Heavy metal resistance of *Streptomyces* isolates

Strain	$NiCl_2$[a]	$NiSO_4$	$AlCl_3$	$CdCl_2$	$CoCl_2$	$CuSO_4$	$ZnSO_4$
Streptomyces coelicolor A3(2)	2.5[b]	<100	0.5	<0.5	<1	<1	<20
Streptomyces mirabilis P16A-1	90	130	1	<0.5	5	2	100
Streptomyces mirabilis K7A-1	70	<100	1	<0.5	4	1	100
Streptomyces chromofuscus P4B-1	50	<100	1	<0.5	10	1	20
Streptomyces mirabilis P10A-3	100	120	1	<0.5	<1	<1	100
Streptomyces chromofuscus P10A-4	50	<100	1	<0.5	2	<1	20
Streptomyces prunicolor P6A-1	50	<100	1	<0.5	<1	1	20
Streptomyces naganishii P9A-1	30	<100	1	<0.5	2	<1	20

[a] All concentrations are given in mmol/l.
[b] Measurement taken from Schmidt et al. (2005).

metal availability of a typical soil from the site where the highly nickel and zinc-resistant strain *S. mirabilis* P16B-1 was isolated (Fig. 2). Sequential extraction revealed that the soil from sample site P16, in addition to the high heavy metal load anticipated, shows a heavy burdening in the bioavailable metal fractions compared to a control soil. Exceptions are the elements iron and manganese. In the case of uranium, the mobile fraction of soil sample P16 contains 0.07 μg/g soil, the specifically adsorbed fraction 438 μg/g while the total content is 1674 μg/g. Thus, the combined bioavailable fractions contain 6258-fold more uranium than the mobile fraction. These exceedingly high results for uranium can be explained by the geology of the former uranium mining site. On a lower level, this phenomenon could also be observed for other elements. When combining the values of both bioavailable fractions, contents of copper were 20-fold higher than in the mobile fraction and those of aluminum 24-fold higher. The factors for the other elements amount to values between 2 and 5. In all cases, the specifically adsorbed fraction contained a higher amount of an element than the mobile fraction indicating the importance of this fraction for bio–geo interactions.

Comparing the results of soil sample P16 with a control soil confirmed the importance of the bioavailable fractions. The total content of nickel, e.g. is four-fold higher in sample P16 but the combined bioavailable fractions showed a 230-fold higher amount than those of a control soil. The strongest difference was found for uranium. The total content of uranium was 290-fold higher in sample P16 but the combined bioavailable fractions contained 27,405-fold higher amounts of uranium than the combined bioavailable fractions of a control soil. The differences between the bioavailable fractions and the total contents clarify the need of an accurate soil analysis when investigating bio–geo interactions in a heavy metal contaminated soil.

A soil sample taken in close vicinity, K7, was used for further plating experiments (Fig. 3), since it showed comparable contamination with the exception of Ni, where the combined bioavailable fractions in sample K7 show a 17% higher contamination.

The data of sequential extraction made it feasible to study the performance of all strains isolated under conditions mimicking the situation by using soil extract media. In such media, only those metals, which can be dissolved by distilled water, are present.

On soil extract agar prepared from contaminated soil, all strains were able to grow, including the control *S. coelicolor* A3(2) (Fig. 3).

In contrast to soil extract, incorporation of soil suspension into the media allows to see effects of both water-soluble and adsorbed metals, if the strains are able to mobilize metals, e.g. by acidification. Indeed, on soil agar, the growth of *S. coelicolor* A3(2) depended on the soil concentration, while the Gessenbach isolates showed good growth even on agar plates with a soil/medium relation of 1:5 (Fig. 3). The pH of soil

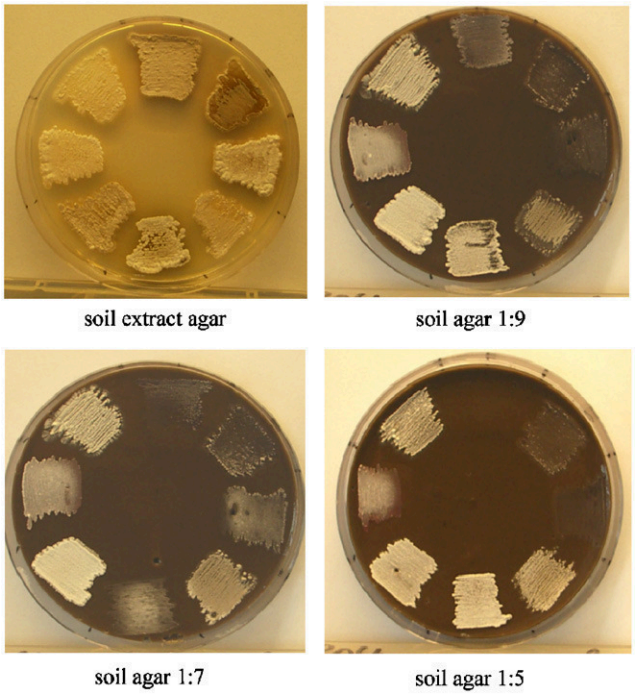

Fig. 3. Growth of highly nickel-resistant streptomycetes and *S. coelicolor* A3(2) on soil agar and soil extract agar. Strains are placed according to Fig. 1.

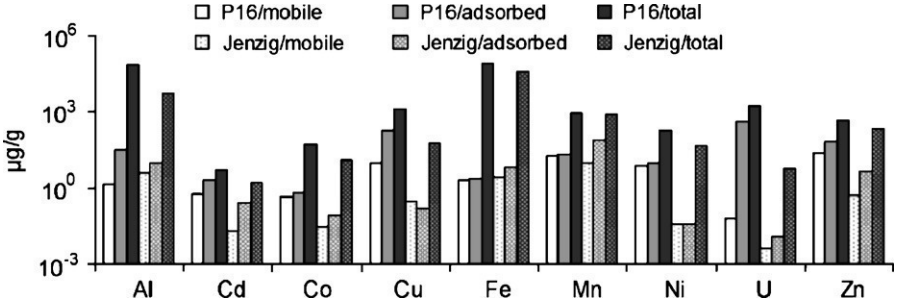

Fig. 2. Data of the sequential extraction. Presented are the mobile fractions, the specifically adsorbed fractions and the total contents of the highly contaminated soil P16 and a control soil.

sample K7 was 5.72 which is not harmful to growth of *S. coelicolor* A3(2).

3.3. Use of different media to elucidate different strategies for nickel resistance

Streptomycetes are well known for their ability to produce a range of secondary metabolites including antibiotics and pigments. It is generally observed that pigment production varies with different growth media. Since sequestration of metals is one strategy for metal tolerance, and since chelating substances include secondary metabolites like melanin, we used a complex medium to test for differences in the identified highly resistant strains *S. mirabilis* P10A-3 and *S. mirabilis* P16B-1 in comparison to cultures grown on minimal medium for Ni resistance (Fig. 4).

S. mirabilis P10A-3 shows a similar growth on nickel-amended TSB medium compared to growth on minimal medium. In contrast, *S. mirabilis* P16B-1 shows reduced growth on 40 mmol/l $NiCl_2$ and is not able to grow on 60 mmol/l or higher concentrations on Ni-TSB plates, while growth on minimal medium is possible at higher Ni concentrations. This unexpected result coincided with differences in the regulation of production of a pigment. Both strains produce a brown pigment likely belonging to the class of melanin (Haferburg et al., 2004) when grown on nickel-amended TSB medium.

S. mirabilis P16B-1 produces a higher amount of this pigment and seemingly is not able to down-regulate the production, whereas *S. mirabilis* P10A-3 stops the production at very high concentrations of nickel. Thus, use of complex media stimulating pigment production interferes with nickel resistance. This observation can be used to distinguish two nickel resistance mechanisms, differential regulation of pigment synthesis vs. resistance mechanisms that are only operating if not countered by pigment production.

S. mirabilis K7A-1 shows a similar result to *S. mirabilis* P16B-1 but was not able to grow on TSB medium amended with 40 mmol/l $NiCl_2$. On 20 mmol/l $NiCl_2$, *S. mirabilis* K7A-1 shows good growth and also produces a brown pigment (data not shown).

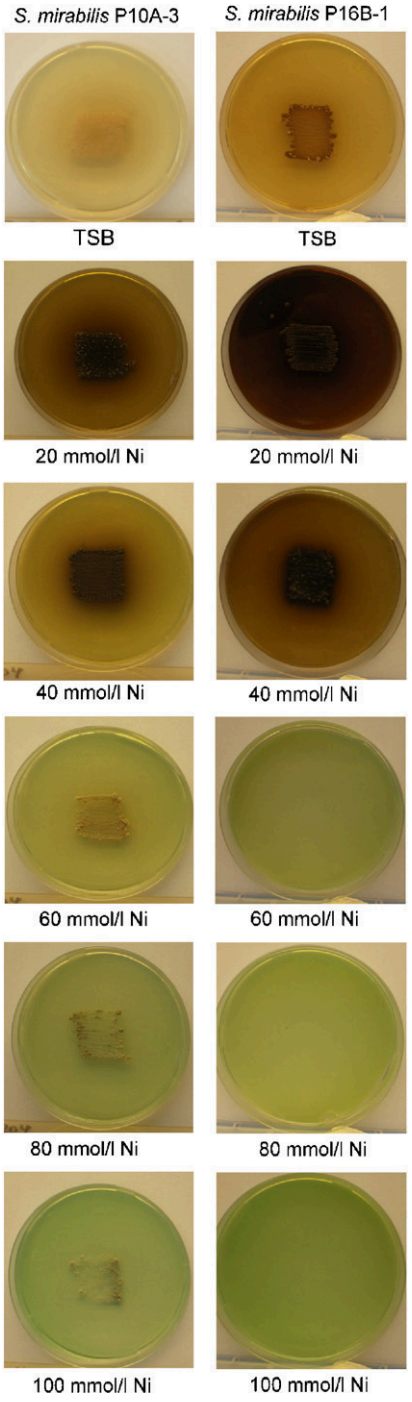

Fig. 4. Comparison of growth of the highly nickel-resistant strains *S. mirabilis* P10A-3 and *S. mirabilis* P16B-1 on nickel-amended TSB media.

3.4. Plasmid content

Plasmids might encode heavy metal efflux transporters that lead to higher resistance. Via PFGE, plasmids could be detected in *S. mirabilis* P16B-1 and *S. mirabilis* K7A-1 (Fig. 5), whereas in the other heavy metal resistant strains, no plasmid could be found. The strain *S. mirabilis* K7A-1 possesses two plasmids, among them one has a similar size to the one detected in *S. mirabilis* P16B-1 (600 kb). The second plasmid in *S. mirabilis* K7A-1 has a size of 200 kb.

Since both strains had shown similar pattern for heavy metal resistances with the difference of *S. mirabilis* K7A-1 not being able to grow on $NiSO_4$ concentrations higher than 100 mmol/l, we conclude that resistance genes might be associated with the plasmid. However, to reveal whether the identified plasmids in both strains actually play a role in heavy

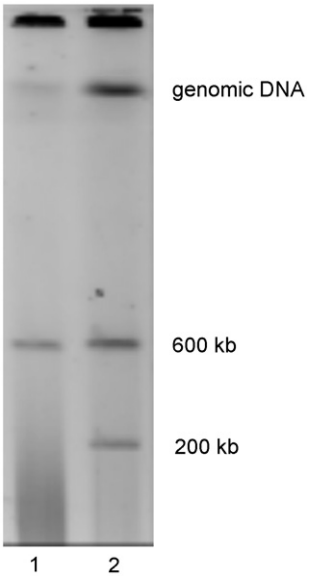

Fig. 5. Pulse-field gel electrophoresis separating plasmids of *S. mirabilis* P16B-1 (1) and *S. mirabilis* K7A-1 (2); kb, kilobases.

metal resistance, further experiments like plasmid curing and plasmid transfer have to be performed.

4. Discussion

Heavy metal resistant bacteria are often found at contaminated sites and in soils with a naturally high amount of heavy metals. Recently, van Nostrand et al. (2007) isolated four actinobacterial strains, among them two of the genus *Streptomyces*, from contaminated riparian sediments. One of the *Streptomyces* strains was able to grow on 85.2 mmol/l nickel which is to the best of our knowledge the highest detected nickel resistance so far. Idris et al. (2004) found plant-associated bacteria in an ultramafic serpentine soil that showed a nickel resistance between 5 and 12 mmol/l. Mengoni et al. (2001) documented that out of 138 bacterial isolates from a serpentine soil, 4% were able to grow on 15 mmol/l nickel.

Soil samples of the banks of the creek Gessenbach contain high amounts of a range of heavy metals as a consequence of mining procedures in this area over several decades. Therefore, the creek Gessenbach appears to be a good location for the search for heavy metal resistant microorganisms. Indeed, bacterial strains of the genus *Streptomyces* that show a high resistance towards nickel occur. From seven analyzed strains, six were able to grow on 50 mmol/l $NiCl_2$, one *S. naganishii* P9A-1 grows on 30 mmol/l $NiCl_2$.

The three strains showing the highest resistances against nickel and also zinc belong to one species, *S. mirabilis*. Nevertheless, they can be clearly separated by their extent of heavy metal resistance and their plasmid equipment. *S. mirabilis* P16B-1 and *S. mirabilis* K7A-1 are isolated from sample sites that are very close to each other. Both show a high resistance to nickel and zinc. *S. mirabilis* P16B-1 possess one plasmid and *S. mirabilis* K7A-1 two, of which the larger is of similar size like the plasmid identified in *S. mirabilis* P16B-1. *S. mirabilis* P10A-3 was found 300 m upstream. It is also resistant to very high concentrations of nickel and zinc. In this strain, no plasmid was found.

Both strains, *S. mirabilis* P16B-1 and *S. mirabilis* P10A-3, performed better on high concentrations of $NiSO_4$ than on $NiCl_2$, indicating that chloride also adds to toxicity, at least for the two strains tested, and that the use of different salts can lead to deviating results which must be taken into account when comparing literature data.

Two further strains with the ability to grow on 50 mmol/l nickel belong to the species *S. chromofuscus*. One of these strains, *S. chromofuscus* P4B-1, also shows a comparatively high resistance towards cobalt.

To the best of our knowledge, resistances against nickel and zinc of over 100 mmol/l have not been described before. Abou-Shanab et al. (2007) tested 45 bacterial isolates from a Ni-rich serpentine soil. Out of 45 strains, 56% were able to grow on 15 mmol/l Ni, but no strain was able to grow on 20 mmol/l Ni. The highest resistance detected in this work for zinc was 10 mmol/l (29%). Resistances against copper and cadmium were much higher, whereas cobalt resistances were in a similar range. Heavy metal resistant *Streptomyces* strains were not described, but other actinobacteria like *Arthrobacter*, *Microbacterium* and *Nocardioides* were identified, most likely owing to different methods of cultivation. However, it should be kept in mind that different working groups use different methods of cultivation, so that the results only can be approximately compared.

Earlier works showed that resistances towards heavy metals can be linked like seen with *C. metallidurans* CH34 (Nies, 2003). For this reason, the nickel-resistant strains isolated from the Gessenbach bank were tested on other heavy metals. A general combination of resistances for all strains could not be detected. However, all three *S. mirabilis* strains are resistant to nickel and zinc, so that it is possible that the underlying resistance mechanism is responsible for detoxification of both heavy metals.

Against cadmium and copper none of the strains showed a remarkable resistance compared with the results of earlier plating experiments (Schmidt et al., 2005). Nevertheless, it should be noted that control strain *S. coelicolor* A3(2) generally is sensitive to heavy metals which is in line with earlier experiments of Abbas and Edwards (1990). All Gessenbach isolates were able

to grow on higher concentrations of nickel, aluminum and zinc.

Soil is much more complex than media amended with heavy metals. It contains a range of heavy metals and different radionuclides, so that it was interesting to analyze whether the results obtained from plating experiments with media correlate with those on soil extract and soil agar. On soil extract agar, all strains including the control strain *S. coelicolor* A3(2) were able to grow. It was concluded that the bioavailable heavy metal content of the soil extract was not high enough for growth inhibition. However, the direct addition of soil to the media did not affect growth of the Gessenbach isolates. Growth of *S. coelicolor* A3(2) was restricted. This leads to the conclusion that adsorbed metals in the soil which could be dissolved by metabolic products of the bacterial strains like excreted acids contribute a sufficient amount of bioavailable contaminants to obtain a critical concentration for the inhibition of heavy metal sensitive strains. This is supported by sequential extractions that show that several metals and radionuclides are bound in the specifically adsorbed fraction.

Plating experiments showed that all Gessenbach isolates were highly resistant to nickel and zinc, and resistant towards aluminum. Either of these metals thus could be responsible for the observed growth inhibition of *S. coelicolor* A3(2) on soil agar. However, in soils of this area also high amounts of uranium are present, which might limit growth of the control strain.

In earlier publications, the use of minimal media is recommended for experiments referring to heavy metal resistance in order to minimize complexation of the heavy metal ions (Amoroso et al., 2000). It is argued that in complex media ingredients could bind heavy metals and thus lower the bioavailability. For this reason, the two strains that showed the highest resistance towards nickel were tested on a complex TSB medium to see whether they can tolerate even higher nickel concentrations on complex media.

While it could be shown that the choice of media can influence the result strongly, and that one strain grew very differently on the chosen media amended with nickel, both strains were not supported in Ni tolerance on this complex medium. *S. mirabilis* P10A-3 showed similar results on both media, but the growth of *S. mirabilis* P16B-1 was strongly reduced on TSB medium plates with high nickel concentrations. These results show that additional ingredients in complex media allow the strains to initiate a synthesis of a brownish pigment (Haferburg et al., 2004). On minimal medium, the synthesis of this pigment does not appear.

S. mirabilis P10A-3 obviously is able to down-regulate this pigment production, while still showing good growth on very high nickel concentrations. This indicates that pigment production is not necessary for growth on high nickel concentrations for this strain.

S. mirabilis P16B-1 does not possess the ability to regulate pigment production and at the same time is not able to grow on TSB plates with highest nickel concentrations tolerated in minimal medium. Since pigment production is energy consuming, less energy is available to operate additional resistance factors like efflux transporters. This might explain the observed lower resistance levels in complex medium. According to these speculations, *S. mirabilis* P10A-3 would be the better adapted strain. The fact that *S. mirabilis* P10A-3 does not contain any plasmid and is able to grow on different media with high concentrations of nickel leads to the conclusion that resistance factors must be located on the chromosome. *S. mirabilis* P16B-1 is not that well adapted. The fact that it contains a plasmid suggests that some of the resistance factors which allow growth on high nickel concentrations in minimal medium could be plasmid-encoded and are not adjusted well to typical defense mechanism like the energy-consuming production of secondary metabolites. These thoughts are confirmed by the fact that strain *S. mirabilis* K7A-1 which shows similar results on nickel-amended TSB medium and which stems from a sample site very close to that of P16B-1 possesses a plasmid of a similar size which could be the same plasmid distributed among strains of this area via plasmid transfer, a process that could be demonstrated for *Streptomyces* strains in earlier works in connection with mercury resistance (Ravel et al., 2000a).

In two *Streptomyces* strains, putative nickel transporters have been identified but it could not be shown whether they are responsible for nickel resistance (Amoroso et al., 2000). Very effective transporters are known from *C. metallidurans* CH34, but because of the differing cell structure with an outer membrane, transporters of gram-negative organisms differ structurally from those of gram-positive organisms. A nickel efflux transporter with a broad host range which also has been found in gram-positive bacteria is NreB (Grass et al., 2001). However, nickel resistance conferred by this transporter amounts to 3 mmol/l nickel (Van Nostrand et al., 2007) but the strains of the creek Gessenbach are able to grow on much higher concentrations. For *S. coelicolor* A3(2), a nickel-responsive regulator (Nur) of the Fur family is known which plays a role in the regulation of nickel homeostasis (Ahn et al., 2006). It regulates a putative nickel transporter gene cluster negatively and thus lowers the influx of nickel when it is present in higher concentrations in the medium. Additionally, it represses the transcription of the *sodF* gene and induces the transcription of the *sodN* gene in the presence of higher nickel concentrations. However, *S. coelicolor* A3(2) is only able to grow on comparatively low concentrations of nickel (Schmidt et al., 2005). Possibly, this is due to the fact that this mechanism could not prevent the influx of nickel ions

through other import systems like such for magnesium (Snavely et al., 1989). On the basis of the known data about nickel resistance in gram-positive bacteria, we assume that a so far unknown high turnover transporter for the high nickel resistance could be responsible.

Apart from transporters, other mechanisms of heavy metal resistance have been indicated. Cadmium-resistant *Streptomyces tendae* F4 obviously excretes a substance that chelates cadmium and allows sensitive strains in its neighborhood to grow on Cd-amended agar (Schmidt et al., 2005). For this reason, bacterial strains in heavy metal contaminated soils do not have to be resistant to all heavy metals, since they can profit from the vicinity of other resistant strains that are able to lower the bioavailability of heavy metals in their surroundings. For *Streptomyces acidiscabies* E13, an intracellular accumulation of nickel in the cytosol was detected (Schmidt et al., 2007), which would be just one example for lowering local metal concentrations.

To the best of our knowledge, only one heavy metal resistance mechanism has been identified on a molecular basis in *Streptomyces* which is the resistance against mercury. However, mercury resistance is based on the reductive detoxification of Hg(II) to elemental, volatile mercury (Ravel et al., 2000b), a mechanism that is not possible for nickel.

To gain more data, further experiments will have to be performed. A promising method is the two-dimensional gel electrophoresis which enables to identify proteins that are induced or repressed under different culture conditions. For *C. metallidurans* CH34, it was possible to identify a range of proteins that are expressed under the influence of different heavy metals, among them regulatory and transport proteins (Noel-Georis et al., 2004).

In addition, the role of the plasmids has to be analyzed. For this purpose, plasmid transfer experiments could support the detection of possible plasmid-encoded heavy metal resistance factors (Ravel et al., 1998).

Acknowledgments

We would like to thank Marc Carlsohn, Monika von der Heide, Ulrike Buhler, Lars Zeggel, Martin Reinicke, Frank Schindler, Eileen Schütze, Christian Dimkpa, Stefan Senitz and Petra Mitscherlich for help. We acknowledge the support of the German Science Foundation through Gk1257.

References

Abbas, A., Edwards, C., 1989. Effects of metals on a range of *Streptomyces* species. Appl. Environ. Microbiol. 55, 2030–2035.

Abbas, A.S., Edwards, C., 1990. Effect of metals on *Streptomyces coelicolor* growth and actinorhodin production. Appl. Environ. Microbiol. 56, 675–680.

Abou-Shanab, R.A.I., van Berkum, P., Angle, J.S., 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. Chemosphere 68, 360–367.

Ahn, B.-E., Joonseok, C., Lee, E.-J., Han, A.-R., Thompson, C.J., Roe, J.-H., 2006. Nur, a nickel responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in Streptomyces coelicolor. Molecular Microbiol 59, 1848–1858.

Amoroso, M.J., Schubert, D., Mitscherlich, P., Schumann, P., Kothe, E., 2000. Evidence for high affinity nickel transporter genes in heavy metal resistant *Streptomyces* spec. J. Basic Microbiol. 40, 295–301.

Anton, A., Große, C., Reißmann, J., Pribyl, T., Nies, D.H., 1999. CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. J. Bacteriol. 181, 6876–6881.

Baath, E., Diaz-Ravina, M., Frostegard, A., Campbell, C.D., 1998. Effect of metal-rich sludge amendments on the soil microbial community. Appl. Environ. Microbiol. 64, 238–245.

Geletneky, J., Paul, M., Merten, D., Büchel, G., 2002. Impact of acid rock drainage in a discrete catchment area at the former uranium mining site Ronneburg (Germany). In: Nelson, J.D., Cincilla, W.A., Foulk, C.L., Hinshaw, L.L., Ketellaper, V. (Eds.), Tailings and Mine Waste, Proceedings of the 9th International Conference on Tailings and Mine Waste, Fort Collins, CO, pp. 67–74.

Grass, G., Fan, B., Rosen, B.P., Lemke, K., Schlegel, H.-G., Rensing, C., 2001. NreB from *Achromobacter xylosoxidans* 31A is a nickel-induced transporter conferring nickel resistance. J. Bacteriol. 183, 2803–2807.

Haferburg, G., Schmidt, A., Reinicke, M., Merten, D., Büchel, G., Kothe, E., 2004. Adaptation to nickel tolerance or nickel resistance in streptomycetes isolated from contaminated soil samples. ISBN 987-21607-0-8.

Hopwood, D.A., 2006. Soil to genomics: the *Streptomyces* chromosome. Annu. Rev. Genet. 40, 2006.

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. Appl. Environ. Microbiol. 70, 2667–2677.

Idris, R., Kuffner, M., Bodrossy, L., Puschenreiter, M., Monchy, S., Wenzel, W.W., Sessitsch, A., 2006. Characterization of Ni-tolerant methylobacteria associated with the hyperaccumulating plant *Thlaspi goesingense* and description of *Methylobacterium goesingense* sp. nov. Syst. Appl. Microbiol. 29, 634–644.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical *Streptomyces* Genetics. The John Innes foundation, Norwich, UK.

Kim, J.-S., Kang, S.-O., Lee, K.J., 2003. The protein complex composed of nickel-binding SrnQ and DNA binding motif-bearing SrnR of *Streptomyces griseus* represses sodF transcription in the presence of nickel. J. Biol. Chem. 278, 18455–18463.

Lopez-Maury, L., Garcia-Dominguez, M., Florencio, F.J., Reyes, J.C., 2002. A two component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. Mol. Microbiol. 43, 247–256.

Mengoni, A., Barzanti, R., Gonnelli, C., Gabbrielli, R., Bazzicalupo, M., 2001. Characterization of nickel-resistant bacteria isolated from serpentine soil. Environ. Microbiol. 3, 691–698.

Mergeay, M., Nies, D., Schlegel, H.D., Gerits, J., Charles, P., van Gijsegem, F., 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162, 328–334.

Mergeay, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., van der Lelie, D., Wattiez, R., 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals towards a catalogue of metal-responsive genes. FEMS Microbiol. Rev. 27, 385–410.

Merten, D., Kothe, E., Büchel, G., 2004. Studies on microbial heavy metal retention from uranium mine drainage water with special emphasis on rare earth elements. Mine Water Environ. 23, 34–43.

Monciardini, P., Sosio, M., Cavaletti, L., Chiocchini, C., Donadio, S., 2002. New PCR primers for the selective amplification of 16S rDNA from different groups of actinomycetes. FEMS Microbiol. Ecol. 42, 419–429.

Nies, D.H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev. 27, 313–339.

Nies, D.H., Mergeay, M., Barbel, F., Schlegel, H.D., 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. 169, 4865–4868.

Noel-Georis, I., Vallaeys, T., Chauvaux, R., Monchy, S., Falmagne, P., Mergeay, M., Wattiey, R., 2004. Global analysis of the *Ralstonia metallidurans* proteome: prelude for the large-scale study of heavy metal response. Proteomics 4, 151–179.

Olafson, R.W., McCubbin, W.D., Cyril, M.K., 1988. Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from a *Synechococcus* sp. cyanobacterium. Biochem. J. 251, 691–699.

Ravel, J., Schrempf, H., Hill, R.T., 1998. Mercury resistance is encoded by transferable giant linear plasmids in two Chesapeake Bay *Streptomyces* strains. Appl. Environ. Microbiol. 64, 3383–3388.

Ravel, J., Wellington, E.M.H., Hill, R.T., 2000a. Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. Appl. Environ. Microbiol. 66.

Ravel, J., DrRuggiero, J., Robb, F.T., Hill, R.T., 2000b. Cloning and sequence analysis of the mercury resistance operon of *Streptomyces* sp. strain CHR28 reveals a novel putative second regulatory gene. J. Bacteriol. 182, 2345–2349.

Raytapadar, S., Datta, R., Paul, A.K., 1995. Effects of some heavy metals on growth, pigment and antibiotic production by *Streptomyces galbus*. Acta Microbiol. Immunol. Hung. 42, 171–177.

Robinson, N.J., Whitehall, S.K., Cavet, J.S., 2001. Microbial metallothioneins. Adv. Microb. Physiol. 44, 183–213.

Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Büchel, G., Kothe, E., 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Geochemistry 65, 131–144.

Schmidt, A., Schmidt, A., Haferburg, G., Kothe, E., 2007. Superoxide dismutases of heavy metal resistant streptomycetes. J. Basic Microbiol. 47, 62–65.

Shevchenko, V., Lisitzin, A., Vinogradova, A., Stein, R., 2003. Heavy metals in aerosols over the seas of the Russian arctic. Sci. Total Environ. 306, 11–25.

Smylla, A., Mroczkowska-Badner, E., 1991. Influence of cadmium ions on *Streptomyces* strains. Acta Microbiol. Pol. 40, 51–58.

Snavely, M.D., Florer, J.B., Miller, C.G., Maguire, M.E., 1989. Magnesium transport in *Salmonella typhimurium*: Mg^{2+} transport by the CorA, MgtA, and MgtB systems. J. Bacteriol. 171, 4761–4766.

Van Nostrand, J.D., Khijniak, T.V., Gentry, T.J., Novak, M.T., Sowder, A.G., Zhou, J.Z., Bertsch, P.M., Morris, P.J., 2007. Isolation and characterization of four gram-positive nickel-tolerant microorganisms from contaminated sediments. Microb. Ecol. 53, 670–682.

Watt, R.K., Ludden, P.W., 1999. Ni(2+) transport and accumulation in *Rhodospirillum rubrum*. J. Bacteriol. 181, 4554–4560.

Youn, H.-D., Kim, E.-J., Roe, J.-H., Hah, Y.-C., Kang, S.-O., 1996. A novel nickel-containing superoxide dismutase from *Streptomyces* spp. Biochem. J. 318, 889–896.

Zeien, H., Bruemmer, G.W., 1989. Chemische Extraktionen zur Bestimmung von Schwermetallbindungsformen in Boeden. Mitt. Dtsch. Bodenkundl. Gesellsch. 59, 505–510.

3.4 *In silico* analysis of nickel containing superoxide dismutase evolution and regulation

Astrid Schmidt, Matthias Gube, André Schmidt and Erika Kothe

Journal of Basic Microbiology (2009) 49, 109–118

Research Paper

In silico analysis of nickel containing superoxide dismutase evolution and regulation

Astrid Schmidt, Matthias Gube, André Schmidt and Erika Kothe

Microbial Phytopathology, Institute of Microbiology, Faculty of Biology and Pharmacy, Friedrich-Schiller-University, Jena, Germany

Superoxide dismutases are essential enzymes involved in detoxification of reactive oxygen by dismutation of the superoxide radical anion. A class of nickel containing superoxide dismutases has been described for streptomycetes and cyanobacteria. *In silico* analysis was used to study the distribution of genes coding for NiSOD in other taxa and to elucidate signals linked to nickel incorporation and maturation of NiSOD. Data mining revealed homologous proteins from actinobacteria, proteobacteria, chlamydiae, and eukarya (green algae) thus allowing a comparison of protein structural elements. Nickel ligands and maturation signals for N-terminal proteolysis were highly conserved. Genomic sequences surrounding genes encoding NiSOD homologs were compared in order to detect putative accessory enzymes involved in maturation. An endopeptidase gene linked to *sodN* coding for NiSOD was found in actinobacteria and cyanobacteria, but not in other taxa. The distribution of NiSOD encoding sequences showed four clusters which are not consistent with the phylogeny of the species. In addition, the different genomic context argues for heterologous gene transfer, most likely from actinobacteria to other taxa. In order to address regulation by nickel availability and incorporation into the mature protein, we present first evidence for putative regulatory nucleotide sequences which will be useful in future studies on nickel uptake and incorporation.

Keywords: Nickel / Superoxide dismutase / Transcriptional regulation / Streptomycetes

Received: September 11, 2008; accepted: October 28, 2008

DOI 10.1002/jobm.200800293

Introduction

Superoxide dismutases are essential enzymes for aerobic organisms since they catalyze the dismutation reaction of superoxide anion radicals in two subsequent half-cycles to yield dioxygen and hydrogen peroxide [1]. This redox reaction is driven by an active center containing a metal ion which alters its redox state during this process [2–4]. Several groups of superoxide dismutases containing different metals can be distinguished. Bimetallic CuZnSOD and FeZnSOD, and the group of MnSOD, FeSOD or cambialistic SOD (using Fe or Mn depending on environmental metal availability [5]) have already been characterized in detail. A third group of nickel-containing SODs, NiSOD, has been described first for the actinobacterial genus *Streptomyces* [6, 7]. Sequence similarities to the *sodN* gene in marine cyanobacterial genomes have also been identified [8–10]. We performed *in silico* analyses to show whether genes with sequence similarity are present outside of these groups thus indicating a more general distribution. Using phylogenetic analyses, we predict a possible evolutionary origin of the enzyme.

NiSOD contains nickel in its active center, and a homohexameric structure with one nickel per subunit has been found [11, 12]. Other nickel containing enzymes have been shown to incorporate nickel into the apoenzyme in a cofactor-associated process [13]. For streptomycete NiSOD, a preprotein with 14 additional N-terminal amino acids is translated. During maturation and

Correspondence: Prof. Dr. Erika Kothe, Mikrobielle Phytopathologie, Institut für Mikrobiologie, Biologisch-Pharmazeutische Fakultät, Friedrich-Schiller-Universität, Neugasse 25, D-07743 Jena, Germany
E-mail: Erika.Kothe@uni-jena.de
Phone: +49 3641 949291
Fax: +49 3641 949292
Internet: www.uni-jena.de/Prof_Dr_Erika_Kothe.html

nickel incorporation, these additional amino acids are cleaved off to form the mature protein, a process which is essential for hexamer formation [6, 11, 12]. The first 9–12 amino acids of the mature NiSOD, designated the nickel hook, have been shown to bind nickel and form the active site. This has recently been addressed in independent approaches by showing SOD activity for either a 9 or 12 amino acid synthetic peptide [14–16].

Nickel containing enzymes usually require a chaperone which mediates the insertion of nickel into the preprotein [17] and, in many cases, protease cleavage co-occurring with nickel insertion has been found to be essential for stable nickel incorporation, as is demonstrated by [NiFe]hydrogenase in *E. coli* [18]. Subsequent to nickel uptake [19], nickel binding factors within the cell are required. A nickel binding protein was identified for *S. seoulensis* [20]. The protein CbiXhp contains a histidine metal binding motif in its C-terminus (13 histidine out of 27 amino acid residues) and one potential CXXC-motif which also might be involved in metal binding. Co-overexpression of *cbiXhp* and *sodN* demonstrated an enhanced activity of NiSOD which was interpreted by CbiXhp functioning as a nickel chelatase during protein maturation.

Known systems which regulate SOD expression in bacteria encompass regulation by oxygen in bacteria that encounter aerobic and anaerobic conditions like *Streptococcus mutans* [5]. This has also been shown for MnSOD encoded by *sodA* in *Escherichia coli* [21]. The FeSOD encoded by *sodB* in *E. coli*, however, is regulated by Fur (ferric uptake regulation protein) [22]. A similar, Fur-like regulation has been shown for *sodF* encoding FeZnSOD of *S. coelicolor* Müller [21, 23, 24]. Nickel seems to provide the regulatory system for SOD expression in streptomycetes.

Promotor elements could be defined which interact with a nickel binding repressor [17] for *sodF1* of *S. coelicolor* A3(2). This *cis*-active region between –60 and +30 was also identified in a region of –2 to +15 in *S. griseus sodF* [25]. This genomic sequence features an inverted repeat (TTGCA) which has been postulated to be a nickel responsive element which binds a nickel sensitive repressor. Similar repeats were found in *sodA* encoding FeSOD of *E. coli* and in *sodA* of *Bacillus subtilis* [25]. In *S. griseus,* the repressor is encoded 3′ to *sodF* by two open reading frames with overlapping stop/start codons. SrnR (for ***s****odF* **r**epression by **n**ickel repressor) shows a similarity to ArsR transcription regulators with a central helix-turn-helix motif whereas the nickel-binding co-repressor SrnQ contains 26% arginine and has no sequence homology to known proteins. Nickel binding leads to conformational changes in the hetero-octamer repressor complex which was postulated to enhance DNA binding affinity [26].

In contrast to *sodF*, *sodN* regulation has been less extensively studied. Regulation by a nickel-sensitive repressor has been postulated [27]. Recently, a nickel responsive regulator (Nur) of the Fur-family has been described for *S. coelicolor*, which binds to the promotor region of *sodF* in the presence of nickel and seems to influence the nickel dependent expression of NiSOD and FeZnSOD [23].

A regulatory system leading to gene expression of either NiSOD or FeZnSOD under different environmental conditions has been identified for streptomycetes [17, 20, 21]. The presence of nickel ions induces the formation of NiSOD if they are present in low micromolar concentrations. On the other hand, FeZnSOD is down-regulated in complex media containing at least 200 μM of $NiCl_2$ [20]. This could also be illustrated in 2D gels after growth of *S. acidiscabies* E13 in minimal medium with 0.3 mM $NiCl_2$ [28]. Both mechanisms take place at the level of transcription [20, 21] in a concentration range of between 10 to 100 μM of nickel. Nickel regulation might be compromised in strains living in nickel-rich environments which led us to analyze specifically nickel resistant strains in previous analyses which were also incorporated into this study [29–31].

Materials and methods

Data mining and statistical analyses

The sequences obtained were investigated using the BLAST tool provided by NCBI genebank and genomes were screened at http://cyano.genome.ad.jp and http://genex2.dri.edu/research/psychro-gen.pdf. Statistical treatments were performed using student's T-tests. Evaluation of database search results and alignments were performed with DNAstar version 4.03.

Phylogenetic analysis

The amino acid sequences were aligned using TCOFFEE 5.13 expresso [32] and MUSCLE 3.6 [33]. Phylogenetic analysis was performed using Neighbor Joining (TREECON v1.3b), Maximum Parsimony (PAUP* v4.0b18 for 32bit Windows, Swofford 2001, Sinauer Associates Inc. Publishers, Sunderland, Massachusetts), Maximum Likelyhood (TREEFINDER v6.07, Jobb 2007) and Bayesian Analysis of Phylogeny (MrBAYES v3.1.2). Evaluation of the Analysis was performed by bootstrap analysis (TREECON, PAUP*), LR-ELW edge support (TREEFINDER), and posterior clade probabilities (MrBAYES).

Protein structure analysis

was done with Deep View/Swiss PdbViewer, v3.7 (Glaxo Smith Kline) by using 1Q0M.pdb [12].

Results

Distribution of NiSOD encoding sequences

We used a bioinformatics approach on sequenced genomes to see whether a unique distribution of NiSOD encoding genes only in streptomycetes and cyanobacteria was supported. To search for yet unidentified NiSOD proteins (EC 1.15.1.1.), BLAST routine was performed using NiSOD from *Streptomyces coelicolor* A3(2) as bait. In addition, the Ni hook (AHCDxxCxxYDP) found at the N-terminus of the enzyme was used for data mining. An unexpectedly high number of new entries were identified for NiSOD homologs. Not only streptomycetes and cyanobacteria but also other actinobacteria such as *Pseudonocardineae*, *Frankineae*, *Propionibacterineae*, *Micromonosporineae*, *Micrococcineae* and *Corynebacterineae* were found to possess sequences with similarity to NiSOD. In addition, flavobacteria, γ- and δ-proteobacteria, chlamydiae, planctomycetes, and even eukaryota (a green algae) were identified (Table 1). However, a similar gene was missing from the genomes of bacilli and other aerotolerant Gram-positives with low GC-content, as well as in further groups of bacteria, in archaea, and in other eukaryote genomes.

Phylogenetic origin of NiSOD

These findings open the discussion on the evolutionary origin of the enzyme. As seen from Table 1, NiSOD encoding sequences were found to be widespread within the actinobacteria. If this group is the likely source of heterologous gene transfer, the members of this group of enzymes should be closely related and cluster together in a phylogenetic tree based on NiSOD sequences. We therefore performed a phylogenetic analysis of NiSOD sequences.

The cladogram of aligned NiSOD protein sequences (Fig. 1) shows well supported clustering into four distinct groups: actinomycetes, cyanobacteria (including one δ-proteobacterium), γ-proteobacteria and flavobacteria together at a third branch, separated from a fourth branch with diverse groups clustering together. We chose an unrooted form with only reliable values over 75 (bootstrap) or 0.95 (MrBayes) as determined by four different methods. The phylogenetically quite diverse fourth group is well supported within the tree but shows a high distance to the other groups in different phylograms. The resulting tree is only partially consistent with bacterial phylogeny based on ribosomal RNA and multilocus analyses. This is indicative of heterologous gene transfer during evolution. The occurrence of NiSOD in all clusters of actinobacteria analyzed suggests the actinobacterial clade as the origin of this enzyme. This assumption was tested by analyses of conservation among putative NiSODs, structural analyses and examination of the genomic context.

Conservation of putative NiSODs

The genomes of different strains of *Prochlorococcus* and *Synechococcus* are available and 13 or six *sodN* sequences, respectively, can be found with highest identity within the genus. Interestingly, *Synechococcus sp.* CC9311 possesses two *sodN* genes (YP_731630 and YP_729969) with only 14.8% amino acid identity. While *SodN1* (YP_729969, Sync_0755) shows identity to the other cyanobacterial NiSODs, *SodN2* (Sync_2434) shows 56.4% identity to *Desulfotalea psychrophila* NiSOD.

The NiSOD homolog of the algae *Ostreococcus tauri,* the smallest (0.95 μm) free-living eukaryotic cell [34], is 366 amino acids long and seems to be a combination of two enzymes. The N-terminus (His1 to Glu151) is similar to the *O. lucimarinus* NiSOD. However, the C-terminus (Met267 to Ser364) shows a higher sequence similarity to BolA, which could be found in direct neighbourhood of the *O. lucimarinus* NiSOD thus indicating a gene fusion during evolution of *O. tauri* (Table 2). BolA of *Escherichia coli* is a morphogene involved in stress response and cell division [35]. BolA-like proteins are widely conserved from prokaryotes to eukaryotes, and seem to be involved in cell proliferation or cell-cycle regulation.

All entries show a highly homologous site at the N-terminus which was described as the proteolytic cleavage site for releasing the N-terminal nickel hook in the mature protein. The putative NiSOD encoding sequences in databases most likely represent genes encoding active NiSOD enzymes because of the good conservation of both the Ni-hook and N-terminal preprotein splice site.

In addition, the sequence similarities of putative helical and structural important regions indicate functional conservation. Only the two algal NiSODs contain an unusually long leader sequence of 67 amino acids with about 72% identity and 31% homology to ubiquitins. Therefore, it might be interesting to investigate whether or not a functional NiSOD can be purified from algae.

Mature forms of experimentally verified and predicted NiSODs consist of 116–151 amino acid residues. The lengths of known and hypothetical N-terminal

Table 1. Species possessing homologs to NiSOD in databases (protein accession numbers).

BACTERIA: Actinobacteria/ Actinomycetales	Streptomycineae	Streptomycetaceae	*Streptomyces avermitilis* MA-4680 (NP_824164)
			Streptomyces coelicolor A3(2) (NP_629400)
			Streptomyces coelicolor Müller (AAC38082)
			Streptomyces seoulensis (AAD17482)
			Streptomyces acidiscabies E13 (ABG24210)
	Pseudonocardineae	Pseudonocardiaceae	*Saccharopolyspora erythraea* NRRL 2338 (YP_001108514)
	Frankineae	Acidothermaceae	*Acidothermus cellulolyticus* 11B (YP_872325)
		Frankiaceae	*Frankia sp.* EAN1pec (ZP_00570998)
	Propionibacterineae	Nocardioidaceae	*Nocardioides* sp. JS614 (YP_922925)
	Micromonosporineae	Micromonosporaceae	*Micromonospora* sp. ATCC 39149 (ABC75844)
			Salinispora arenicola CNS205 (ZP_01649012)
			Salinispora tropica CNB-440 (YP_001160506)
	Micrococcineae	Intrasporangiaceae	*Janibacter* sp. HTCC2649 (ZP_00993682)
	Corynebacterineae	Mycobacteriaceae	*Mycobacterium vanbaalenii* PYR-1 (YP_956442)
			Mycobacterium gilvum PYR-GCK (YP_001132405)
Chlamydiae/ Lentisphaerae	Victivallales	Victivallaceae	*Victivallis vadensis* ATCC BAA-548 (ZP_01924586.1)
	Lentisphaerales	Lentisphaera	*Lentisphaera araneosa* HTCC2155 (ZP_01874658)
Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Polaribacter irgensii* 23-P (ZP_01118615)
			Psychroflexus torquis ATCC 700755 (ZP_01254046)
Delta-proteobacteria	Desulfobacterales	Desulfobacteriaceae	*Desulfotalea psychrophila* LSv54 (YP_066240)
	Myxococcales	Nannocystaceae	*Plesiocystis pacifica* SIR-1 (ZP_01909245)
Gamma-proteobacteria	Alteromonadales	Colwelliaceae	*Colwellia psychrerythraea* 34H (YP_267202)
		Shewanellaceae	*Shewanella woodyi* ATCC 51908 (ZP_01540983)
		Moritellaceae	*Moritella sp.* PE36 (ZP_01897719)
	Oceanospirillales		*Marinomonas* sp. MWYL1 (ZP_01596972)
			Marinomonas sp. MED121 (ZP_01074452)
Cyanobacteria	Prochlorales	Prochlorococcaceae	*Prochlorococcus marinus str.* AS9601 (YP_001009883)
			Prochlorococcus marinus str. MED4 (PMM1294)
			Prochlorococcus marinus str. MIT 9211 (ZP_01004940)
			Prochlorococcus marinus str. MIT 9301 (YP_001091703)
			Prochlorococcus marinus str. MIT 9303 (YP_001017980)
			Prochlorococcus marinus str. MIT 9312 (YP_397886)
			Prochlorococcus marinus str. MIT 9313 (NP_894173)
			Prochlorococcus marinus str. MIT 9515 (YP_001011769)
			Prochlorococcus marinus str. NATL1A (YP_001015534)
			Prochlorococcus marinus str. NATL2A (YP_292055)
			Prochlorococcus marinus str. SS120 (Pro1368)
			Prochlorococcus marinus subsp. marinus str. CCMP1375 (NP_875759)
			Prochlorococcus marinus subsp. pastoris str. CCMP1986 (NP_893411)
	Chroococcales		*Crocosphaera watsonii* WH 8501 (ZP_00517273)
			Synechococcus sp. BL107 (ZP_01469600)
			Synechococcus sp. CC9311 (YP_731630 and YP_729969)
			Synechococcus sp. CC9902 (YP_377527)
			Synechococcus sp. CC9605 (YP_381196)
			Synechococcus sp. *WH8102* (NP_897719)
	Oscillatoriales		*Trichodesmium erythraeum* IMS101 (YP_720765)
Planctomycetes	Planctomycetales	Planctomycetaceae	*Rhodopirellula baltica* SH 1 (NP_870618)
EUKARYA Chlorophyta	Mamiellales	Mamiellaceae	*Ostreococcus lucimarinus* CCE9901 (XP_001415976)
			Ostreococcus tauri (CAL50443)

leader peptides range between 12 and 67 residues. Alignment of the entries (Fig. 2) shows that in addition to the Ni-hook eight amino acids are completely conserved and in their surrounding 11 amino acids are found with up to 3 amino acid exchanges. These highly homologous regions could indicate amino acids essential for forming the tertiary structure, subunit interactions of the protein, nickel binding and incorporation, or for the channeling of superoxide molecules to the active center.

More specifically, His1, Cys2, Cys6 are assumed to be involved in nickel coordination. These are completely conserved in accordance with functional requirements [12, 36, 37].

Asp3, or Pro5, or Tyr9 are postulated to donate protons. Most conceptual translation products carry Asp3

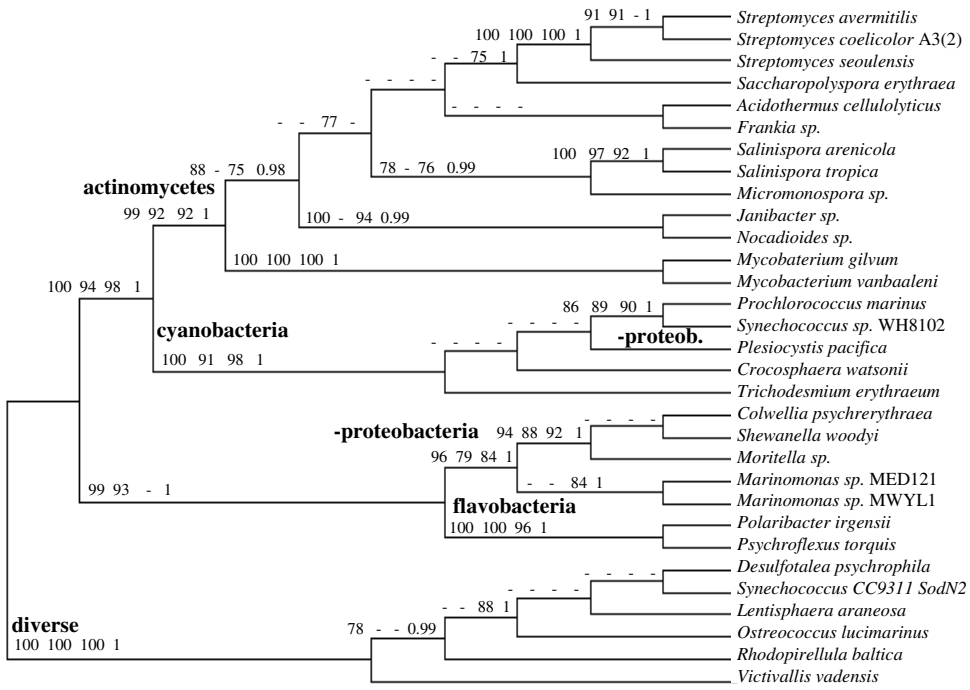

Figure 1. Unrooted cladogram of 31 NiSOD protein sequences calculated by TREEFINDER v6.07 based on TCOFFEE 1.53 expresso alignment. Edge support values are given in the order TREECON v1.3b, PAUP* v4b10, TREEFINDER v6.07, MrBAYES v3.1.2 based on the same alignment. Only values over 75 (TREECON, PAUP*, TREEFINDER) or 0.95 (MrBAYES) were taken into account.

which is discussed as a proton donor. However, *Desulfotalea*, *Synechococcus* SodN2, chlamydiae, planctomycetes and algae contain a Gln or Glu at this position. Thus, the presumed function of Asp3 needs to be addressed by mutational analysis. Pro5 might contribute to channel construction together with Tyr9 and Asp61. Pro5 is postulated to constitute a hinge residue, and is also strongly conserved. It is only exchanged for Phe5 in *Mycobacterium gilvum* and to Tyr5 in *M. vanbaalenii*. Tyr9 is thought to be essential for channel constriction. It is generally conserved, with an exchange for Phe9 only in the putative plant gene products.

For Gly7 no function has been assigned. It is exchanged for Lys7 in all γ-proteobacteria.

Val8 interacts, together with Glu17 with His1, in the reduced state of NiSOD and blocks backside access to the Ni ion. It is conserved only in actinomycete and cyanobacterial NiSOD, all other contain Ile8 which might be a functional substitution. Glu17 is found only in actinomycetes and *R. baltica* NiSOD.

Asp10 is exchanged to Asn10 in *D. psychrophila* and to Gly10 in *R. baltica*, while it is duplicated in *Synechococcus* SodN2, both algal and the chlamydiae NiSODs (Asp10, Asp11). Pro11 is exchanged to Asp11 in *D. psychrophila* and *R. baltica*. Thus, all NiSODs clustering in the third branch (see Fig. 1) carry Asp11, with only the algal gene product possessing Pro11.

Genomic context of NiSOD

The genomic surrounding of *sodN* was screened to identify genes encoding accessory proteins involved in nickel homeostasis and incorporation. Indeed, homologous proteins were found to be encoded adjacent to the *sodN* gene (Table 2). Putative endopeptidases in the neighbourhood of *sodN* are found in all actinomycetes, γ-proteobacteria and cyanobacteria. All of these peptidases contain a special amino acid sequence at their N-terminus, with sequence similarity to an endopeptidase in plants *(Arabidopsis thaliana)*. While both are divergently transcribed from an intergenic region in actinomycetes, the endopeptidase gene is located downstream of *sodN* in γ-proteobacteria and cyanobacteria. The change of relative transcription direction between the endopeptidase and *sodN* could be indicative of an independent acqisition of the two proteins involved in expression of active NiSOD, or inversion events forced by different control mechanisms.

Only *Prochlorococcus* and *Synechococcus* (not the cyanobacterium *T. erythraeum*) feature a further potential nickel incorporation protein one place apart from the endopeptidase (see Table 2). The cyanobacterial genes show a sequence similarity of 63.2% and have about 28–30% similarity to plant high affinity nickel transporter genes, e.g. *O. lucimarinus* (OSTLU_43009) and

Table 2. Genomic region around sodN and direction of ORFs. Shadowed boxes indicate sequence similarities, and intergenic region lenght between *sodN* homolog and putative maturation endopeptidase gene is given.

			nt		
S. coelicolor A3(2)		Sco5255 – endopeptidase	142	*sodN* +	Sco5253 – hypothetical protein
S. avermitilis		Sav2987 -endopeptidase	85	*sodN* + NiSOD	Sav2989 – MarR-family transcriptional regulator
Saccharopolyspora erythraea NRRL 2338	SACE_6417 – pteG cholesterol oxidase	SACE_6418 – hypothetical protein	135	SACE_6419 + *sodN*	SACE_6420 + transcriptional regulator, DNA binding domain at their C-terminal.
Acidothermus cellulolyticus 11B	Acel_0568 + dTDP-4-dehydrorhamnose 3,5-epimerase	Acel_0567 – endopeptidase protein	108	Acel_0566+ superoxide dismutase	Acel_0565 + anti-sigma regulatory factor, serine/threonine protein kinase
Nocardioides sp. JS614	Noca_1727 – Malate dehydrogenase	Noca_1726 – Peptidase_S24	87	Noca_1725 + nickel-containing SOD	Noca_1724 – phage integrase phiLC3
Salinispora tropica CNB-440	Strop_3695 – Malate dehydrogenase	Strop_3696 – Peptidase_S24	25	Strop_3697 + nickel-containing SOD	Strop_3698 + hypothetical protein
Mycobacterium vanbaalenii PYR-1	Mvan_5669 – Rhodanese domain protein	Mvan_5670 – Peptidase_S24	22	Mvan_5671 + nickel-containing SOD.	Mvan_5672 + hypothetical protein
Mycobacterium gilvum PYR-GCK	Mflv_1137 + hypothetical protein	Mflv_1136 – putative phage repressor	68	Mflv_1135 + Nickel-containing SOD	Mflv_1134 + hypothetical protein
Desulfotalea psychrophila LSv54		DP2505 – hypothetical protein		DP2504 + nickel-containing superoxide dismutase.	DPt45 – tRNA-Glu
Ostreococcus lucimarinus CCE9901		OSTLU_29163 – predicted protein		OSTLU_29162 + nickel-containing superoxide dismutase.	OSTLU_29161 + BolA; Stress-induced signal transduction mechanisms
Rhodopirellula baltica SH 1 [RB12632/5 not occupied]	RB12630 + protein-signal peptide	RB12631 + protein-signal peptide, transmembrane metal-dependent hydrolase of the TIM-barrel fold	RB12633 + Cupin_4; cupin superfamily protein	RB12634 – Nickel-containing superoxide dismutase.	RB12636 – acetyl-CoA carboxylase, ATP-grasp enzyme
Colwellia psychrerythraea 34H		CPS_0445 – transcriptional regulator	22	CPS_0444 – nickel-containing SOD	CPS_0443 – hypothetical protein
Trichodesmium erythraeum IMS101	Tery_0893 – heat shock protein 70	Tery_0892 – hypothetical protein	31	Tery_0891 – nickel-containing SOD	Tery_0890 – CysZ; cysteine biosynthesis
Prochlorococcus marinus subsp. pastoris str. CCMP 1375 (conserved in *Prochlorococcus*)	Pro1370 + hydrogenase accessory protein or high-affinity nickel-transport protein	Pro1369 – endopeptidase I	28	Pro1368 – Nickel-containing SOD	Pro1367 – FKBP-type petodyl-prylyl-cis-trans-isomerase
Synechococcus sp. WH8102 (conserved in *Synechococcus*)	Synw1628 + cyanobacterial conserved hypothetical protein	Synw1627 – peptidase S26 family protein	11	Synw1626 – nickel containing SOD	Synw1625 – FKBP-type petodyl-prylyl-cis-trans-isomerase

A. thaliana (AT4G35080). These proteins can be considered as being additional putative nickel access and nickel incorporation proteins.

At the 5′ end of *sodN*, both cyanobacterial genera contain an isomerase encoding gene (62.1% sequence identity) while in several actinomycetes putative tran-

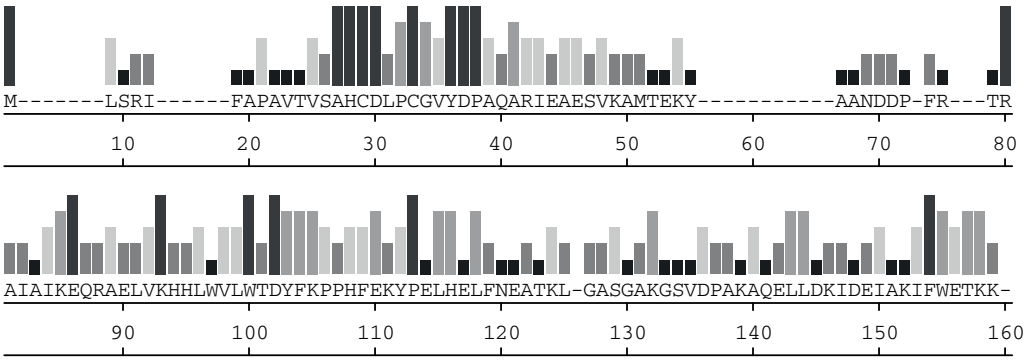

Figure 2. Alignment report of 25 NiSOD sequences. The histogram of match strength is given as indicator for conservation (darker color for higher match strength).

scriptional regulators are found. These, however, lack sequence identity among each other. Two hypothetical proteins of both *Mycobacterium* sp. show additional similarities. Further investigation of a possible role of the transcriptional regulators in nickel incorporation or homeostasis will be necessary to show whether this might be one of the missing factors in transcriptional regulation of NiSOD expression.

Identification of regulatory sequences in SOD genes

Relating to the homologies, the spacer sequences between endopeptidase genes and *sodN* were compared. Although they generally differ in sequence and length, all *Streptomyes* spacers share 86–90% sequence identity. This indicates common evolution and functional conservation within this clade of bacteria.

Comparing the sequences surrounding *sodN*, a conserved motif of 19 bp length in all *Streptomyces* genomes was discovered (TGA GAA GAC GAT CAC GAG G), which is located at the transcription start site (–1) for all *sodN* genes. The same sequence can be found in inverse orientation downstream of all known *sodF* genes (Fig. 3) with a distance of 14 to 25 nucleotides to the translational stop codon. This sequence is present in all messengers encoding streptomycete superoxide dismutases, regardless of whether these are NiSOD or FeZnSOD. Thus, these sequence elements might possibly be either transcriptional regulators, or else translational regulatory elements since they are present within the transcription product. At this point, further studies on the function of these sequence elements are needed in order to reveal whether they are indeed involved in SOD gene expression regulation.

Discussion

Several homologs in bacteria and green algae could be identified and we expect even more homologs as more database entries become available. In a recent study, Dupont *et al.* [38] could already identify sequences for nickel containing superoxide dismutases from environmental, metagenome analyses and clustered them within a cladogram of the genomic SodN sequences available. However, two different cladograms, both with extremely low bootstrap supports on the higher

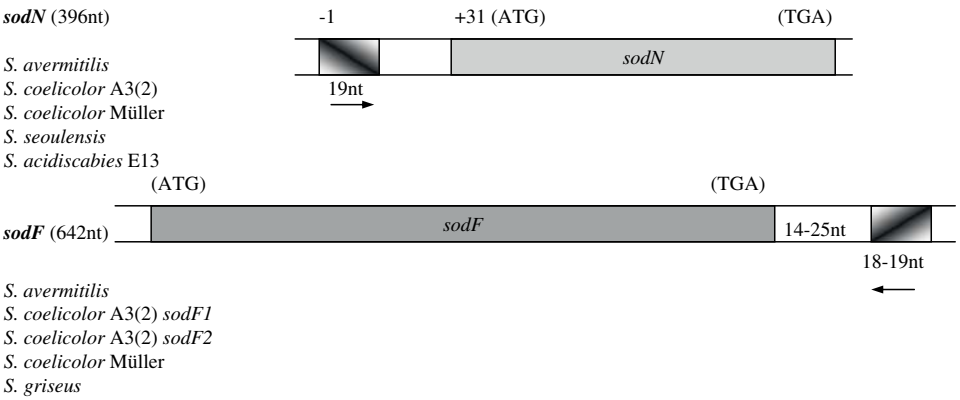

Figure 3. Localization of the putative 19 bp regulatory element with respect to *sodN* and *sodF* genes in streptomycetes.

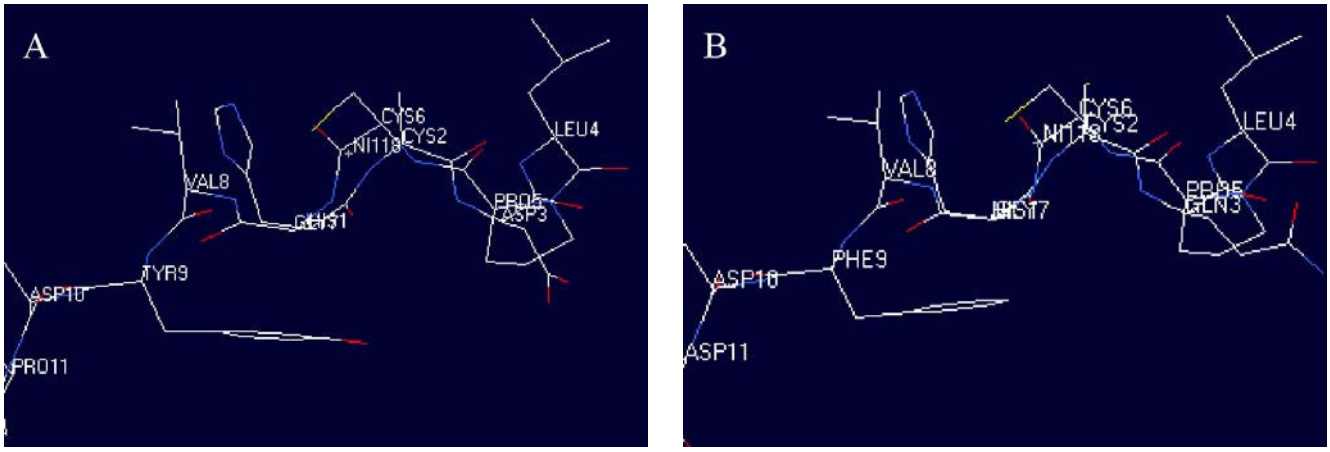

Figure 4. Comparison of Ni-hook structure of *S. seoulensis* (A) and *O. lucimarinus* (B) with focus at the angle of F/Y9.

order branches were shown. In our cladogram, we used four different indicators of phylogenetic distance and show very high bootstrap supports for all major branches in our tree.

The gene *sodN* is present in all actinomycetes we investigated and is missing in members of the other clades thus suggesting actinobacteria to be the source of heterologous gene transfer. This gene transfer most likely took place in marine environments as most members with acquired *sodN* homologs are marine organisms. The marine environment provides the organisms with the same nickel and low iron availabilities which may have supported heterologous gene transfer rather than being indicative of vertical gene evolution. The world's smallest free-living eukaryote *O. tauri* possesses a putative NiSOD::BolA fusion protein, a feature which has been described for this organism with extremely high gene density through gene fusion [36]. In this protein the α3 and α4 helices are missing thus questioning NiSOD activity of this gene product. Comparison of the 3D-structure of the algal Ni-hook shows a similar, but slightly rotated orientation of the lid Tyr9 or Phe9 as well as a missing OH-group (Fig. 4). Taken together, these changes challenge the function of the algal protein as NiSOD.

All NiSOD encoding genes show the proteolytic cleavage site (with a Gly or a Ser instead of Ala preceeding His1 in chlorophyta and *Synechococcus* SodN2, respectively; see Table 3). Functions attributed to amino acids within the nickel hook have been discussed and evidence for an inner sphere mechanism of catalysis was given in contrast to earlier publications [see 15, 16 and references therein]. This places an added interest in the nickel hook and the conservation analysis provided here. Interestingly, a novel protein of unknown function, DUF1619 of *Xenopus tropicalis,* contains a Ni-hook-similar sequence (AHCDDNCCC-DP) which turned up in our investigation which should be analyzed for function in future studies.

In addition to further experimental evidence supporting the roles of single amino acids in the SOD function of the nickel hook, further investigation of the bulk of the protein needs to be performed in order to show whether structural differences, subunit association differences, or differences in the part of the bulk protein forming a guiding channel for the superoxide anion radical are observed between the different homologs.

In addition to residues contributing to Ni incorporation, enzyme folding and assembly, three conserved lysine residues have been proposed to form an electrostatic guidance system to the active site, reminescent of other SODs. For *S. coelicolor* Lys64, Lys115 and Lys27C have been assigned such a function [11, 12]. Lys27C is

Table 3. Ni-hook identities in different phylogenetic groups (number of different species), see text.

	A	H1	C2	D3	x	x	C6	x	x	Y9	D10	P11
actinomycetes (13)	A	H	C	D	L	P (L/Y)	C	G	V	Y	D	P
cyanobacteria (4)	A	H	C	D	L	P	C	G	V	Y	D	P
γ-proteobacteria (5)	A	H	C	D	I	P	C	K	I	Y	D	P
flavobacteria (2)	A	H	C	D	I	P	C	G	I	Y	D	P
diverse (7)	A (G/S)	H	C	Q/E	V (L/I)	P	C	G	I	Y (F)	D (G/N)	D

conserved in most NiSODs, however, all γ-proteobacteria and flavobacteria possess Leu27. Lys64 is exchanged for Thr64 in both *Mycobacterium,* but *M. vanbaaleni* carries Lys65. Lys115 is conserved in all actinomycetes, cyanobacteria, flavobacteria, *P. pacifica* and all γ-proteobacteria, with both *Mycobacteria* carrying Asp115 and Lys116. This strong, but not absolute conservation warrants experimental investigation of the function of these lysine residues as substrate guidance systems in enzymatic function.

As mentioned above, accessory proteins play a vital role for NiSOD assembly. In all but δ-proteobacteria, planctomycetes and chlorophyta genomes, a putative maturation endopeptidase is located adjacent to the NiSOD encoding gene. The relevance of the maturation endopeptidase for NiSOD expression could be shown by transformation of *E. coli* with *sodN,* which yielded functional SOD only by co-transformation with the adjacent maturation endopeptidase gene [8].

Our data mining revealed a 19 bp motive found exclusively in *Streptomyces* genomes which is located at the transcription start site of all NiSOD encoding genes, and found inversely oriented downstream of all known SodF encoding genes. This sequence is interpreted to be a transcriptional/translational regulatory element. It is present within the transcription product for both NiSOD and FeZnSOD. None of the non-actinobacterial organisms which lack this regulatory element contain a FeZnSOD thus suggesting a role in NiSOD *versus* FeZnSOD regulation of streptomycetes. A putative role as transcriptional regulator will have to be verified experimentally in future investigations. The use of strains adapted to high nickel concentrations in the environment [31, 39] (see Haferburg *et al.* 2007, Schmidt *et al.* 2008a) will aid such investigations.

Acknowledgements

We would like to thank Deutsche Forschungsgemeinschaft (DFG) for funding and Petra Mitscherlich for technical assistance.

References

[1] Fridovich, I., 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem., **64**, 97–112.

[2] Choudhury, S.B., Lee, J.W., Davidson, G., Yim, Y.I., Bose, K., Sharma, M.L., Kang, S.-O., Cabelli, D.E. and Maroney, M.J., 1999. Examination of the nickel site structure and reaction mechanism in *Streptomyces seoulenis* superoxide dismutase. Biochemistry, **38**, 3744–3752.

[3] Pelmenschikov, V. and Siegbahn, P.E.M., 2006. Nickel superoxide dismutase reaction mechanism studied by hybrid density functional methods. J. Am. Chem. Soc., **128**, 7466–7475.

[4] Szilagyi, R.K., Bryngelson, P.A., Maroney, M.J., Hedman, B., Hodgson, K.O. and Solomon, E.I., 2004. S K-edge X-ray absorption spectroscopic investigation of the Ni-containing superoxide dismutase active site: new structural insight into the mechanism. J. Am. Chem. Soc., **126**, 3018–3019.

[5] Martin, M., Byers, B.R., Olson, M.O.J., Salin, M.L., Arceneaux, J.E.L. and Tolbert C., 1986. A *Streptococcus mutans* superoxide dimutase that is active with either manganese or iron as cofactor. J. Biol. Chem., **261**, 9361–9367.

[6] Youn, H.-D., Kim, E.-J., Roe, J.-H., Hah, Y.C. and Kang, S.-O., 1996a. A novel nickel-containing superoxide dismutase from *Streptomyces spp*. Biochem. J., **318**, 889–896.

[7] Youn, H.-D., Youn, H., Lee, J.-W., Yim, Y.-I., Lee, J.K., Hah, Y.C. and Kang, S.-O., 1996b. Unique isoenzymes of superoxide dismutase in *Streptomyces griseus*. Arch. Biochem. Biophys., **334**, 341–348.

[8] Eitinger, T., 2004. *In vivo* production of active nickel superoxide dismutase from *Prochlorococcus marinus* MIT9313 is dependent on its cognate peptidase. J. Bacteriol., **186**, 7821–7825.

[9] Palenik, B., Brahamsha, B., Larimer, F.W., Land, M., Hauser, L. *et al*., 2003. The genome of a motile marine *Synechococcus*. Nature, **424**, 1037–1042.

[10] Priya, B., Premanandh, J., Dhanalakshmi, R.T., Seethalakshmi, T., Uma, L., Prabaharan, D. and Subramanian, G., 2007. Comparative analysis of cyanobacterial superoxide dismutases to discriminate canonical forms. BMC Genomics, **8**, 1–10.

[11] Barondeau, D.P., Kassmann, C.J., Bruns, C.K., Tainer, J.A. and Getzoff, E.D., 2004. Nickel superoxide dismutase structure and mechanism. Biochemistry, **43**, 8038–8047.

[12] Würges, J., Lee, J.W., Yim, Y.I., Yim, H.S., Kang, S.O. and Carugo, K.D., 2004. Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. Proc. Natl. Acad. Sci. USA, **101**, 8569–8574.

[13] Watt, R.K. and Ludden, P.W., 1999. Nickel-binding proteins. Cell Mol. Life Sci., **56**, 604–625.

[14] Shearer, J. and Long, L.M., 2006. A nickel superoxide dismutase maquette that reproduces the spectroscopic and functional properties of the metalloenzyme. Inorg. Chem., **45**, 2358–2360.

[15] Tietze, D., Breitzke, H., Imhof, D., Kothe, E., Weston, J. and Buntkowsky, G., 2009. New insight into the mode of action of nickel superoxide dismutase by investigating metallopeptide substrate models. Chemistry, **15**, 517–523.

[16] Schmidt, M., Zahn, S., Carella, M., Öhlenschläger, O., Görlach, M., Kothe, E. and Weston, J., 2008. Solution structure of a functional biomimetic and mechanistic implications for nickel superoxide dismutases. Chem. Bio. Chem., **9**, 11–13.

[17] Chung, H.-J., Choi, J.-H., Kim, E.-J., Cho, Y.-H. and Roe, J.-H., 1999. Negative regulation of the gene for Fe-containing superoxide dismutase by an Ni-responsive factor in *Streptomyces coelicolor*. J. Bacteriol., **181**, 7381–7384.

[18] Blokesch, M., Paschos, A., Theodoratou, E., Bauer, A., Hube, M., Huth, S. and Böck, A., 2002. Metal insertion into NiFe-hydrogenases. Biochem. Soc. Transact., **30**, 674–680.

[19] Rodionov, D.A., Hebbeln, P., Gelfand, M.S. and Eitinger, T., 2006. Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: Evidence for a novel group of ATP-binding cassette transporters. J. Bacteriol., **188**, 317–327.

[20] Kim, E.-J., Chung, H.-J., Suh, B., Hah, Y.C. and Roe, J.-H., 1998b. Expression and regulation of the *sodF* gene encoding iron- and zinc-containing superoxide dismutase in *Streptomyces coelicolor* Müller. J. Bacteriol., **180**, 2014–2020.

[21] Kim, E.-J., Chung, H.-J., Suh, B., Hah, Y.C. and Roe, J.-H., 1998a. Transcriptional and post-transcriptional regulation by nickel of *sodN* gene encoding nickel-containing superoxide dismutase from *Streptomyces coelicolor* Müller. Mol. Microbiol., **27**, 187–195.

[22] Compan, I. and Touati, D., 1993. Interaction of six global transcription regulators in expression of manganese superoxide dimutase in *Escherichia coli* K12. J. Bacteriol., **175**, 1687–1696.

[23] Ahn, B.E., Cha, J., Lee, E.J., Han, A.R., Thompson, C.J. and Roe, J.H., 2006. Nur, a nickel-responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in *Streptomyces coelicolor*. Mol. Microbiol., **59**, 1848–1858.

[24] Hahn, J.-S., Oh, S.-Y. and Roe, J.-H., 2000. Regulation of the *furA* and *catC* operon, encoding a ferric uptake regulator homolog and catalase-peroxidase, respectively, in *Streptomyces coelicolor* A3(2). J. Bacteriol., **275**, 3767–3774.

[25] Kim, E.J., Jang, J.-H., Lee, J.-W., Kang, S.-O., Kim, K.-S. and Lee, J.K., 2000. Identification of cis site involved in nickel-responsive transcriptional repression of *sodF* gene for Fe- and Zn-containing superoxide dismutase of *Streptomyces griseus*. Biochim. Biophys. Acta, **1493**, 200–207.

[26] Kim, I.K., Yim, Y.I., Kim, J.M., Lee, J.W., Yim, H.S. and Kang, S.O., 2003a. CbiX-homologous protein (CbiXhp), a metal-binding protein, from *Streptomyces seoulensis* is involved in expression of nickel-containing superoxide dismutase. FEMS Microbiol. Lett., **228**, 21–26.

[27] Kim, J.S., Kang, S.O. and Lee, J.K., 2003b. The protein complex composed of nickel-binding SrnQ and DNA binding motif-bearing SrnR of *Streptomyces griseus* represses *sodF* transcription in the presence of nickel. J. Biol. Chem., **278**, 18455–18463.

[28] Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Büchel, G. and Kothe, E., 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Chemie Erde, **65**, 131–144.

[29] Amoroso, M.J., Schubert, D., Mitscherlich, P., Schumann, P. and Kothe, E., 2000. Evidence for high affinity nickel transporter genes in heavy metal resistant *Streptomyces* spec. J. Basic Microbiol., **40**, 295–301.

[30] Schmidt, A., Schmidt, A., Haferburg, G. and Kothe, E., 2007. Superoxide dismutases of heavy metal resistant streptomycetes. J. Basic Microbiol., **47**, 56–62.

[31] Schmidt, A., Haferburg, G., Schmidt, A., Lischke, U., Merten, D., Gherghel, F., Büchel, G. and Kothe, E., 2008a. Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. Chem. Erde, DOI 10.1016/j.chemer.2007.II.002.

[32] Armougom, F., Moretti, S., Poirot, O., Audic, S., Dumas, P., Schaeli, B., Keduas, V. and Notredame, C., 2006. Expresso: automatic incorporation of structural information in multiple sequence alignments using 3D-Coffee. Nucleic Acids Res., **34**, W604–608.

[33] Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucl. Acids Res., **32**, 1792–1797.

[34] Rodríguez, F., Derelle, E., Guillou, L., Le Gall, F., Vaulot, D. and Moreau, H., 2005. Ecotype diversity in the marine picoeukaryote *Ostreococcus* (Chlorophyta, Prasinophyceae). Environ. Microbiol., **7**, 853–859.

[35] Derelle, E., Ferraz, C., Rombauts, S., Rouzé, P., Worden, *et al.*, 2006. Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc. Natl. Acad. Sci. USA, **103**, 11647–11652.

[36] Fiedler, A.T., Bryngelson, P.A., Maroney, M.J. and Brunold, T.C., 2005. Spectroscopic and computational studies of Ni superoxide dismutase: Electronic structure contributions to enzymatic function. J. Am. Chem. Soc., **127**, 5449–5462.

[37] Neupane, K.P. and Shearer, J., 2006. The influence of amine/amide versus bisamide coordination in nickel superoxide dismutase. Inorg. Chem., **45**, 10552–10566.

[38] Dupont, C.l., Neupane, K., Shearer, J. and Palenik, B., 2008. Diversity, fungtion and evolution of genes coding for putative Ni-containing superoxide dismutases. Environ. Microbiol., **10**, 1831–1843.

[39] Haferburg, G., Reinicke, M., Merten, D., Büchel, G. and Kothe, E., 2007. Microbes adapted to acid mine drainage as source for strains active in retention of aluminium or uranium. J. Geochem. Expl., **92**, 196–204.

3.5 *In silico* prediction of potential metallothioneins and metallohistins in actinobacteria

Andre Schmidt, Matthias Hagen, Eileen Schütze, Astrid Schmidt and Erika Kothe

Journal of Basic Microbiology, accepted (2010)

Research Paper

In silico prediction of potential metallothioneins and metallohistins in actinobacteria

Andre Schmidt[1], Matthias Hagen[2], Eileen Schütze[1], Astrid Schmidt[1] and Erika Kothe[1]

[1] Microbial Phytopathology, Institute of Microbiology, Faculty of Biology and Pharmacy, Friedrich-Schiller-University, Jena, Germany
[2] Web Technology & Information Systems, Faculty of Media, Bauhaus University, Weimar, Germany

Metallothioneins and metallohistins are short peptides with a high cysteine and/or histidine content able to coordinate metals intracellularly, thereby increasing the tolerance against elevated concentrations of metals. Because of their features, they can be detected by *in silico* prediction from proteomes annotated from sequenced genomes. Here, we analyzed 73 sequenced actinobacterial genomes for peptides (≤100 amino acids) with a high content of cysteine and histidine (≥15%) and identified 103 putative metallothioneins and metallohistins. For 45 of these peptides, we found similarities to metal binding protein domains, including zinc fingers, heavy metal transporters or eukaryotic metallothioneins, which can serve as proof-of-principle in underscoring a potential function as metal binding peptides. An evolutionary origin from metal containing domains of enzymes is discussed and metallohistins not containing cysteine are described for the first time for bacteria.

Supporting Information for this article is available from the authors on the WWW under http://www.wiley-vch.de/contents/jc2248/2010/201000055_s.pdf

Keywords: Metallothionein / Metallohistin / Actinobacteria / Heavy metal resistance / *In silico*

Received: February 09, 2010; accepted: June 29, 2010

DOI 10.1002/jobm.201000055

Introduction

Metallothioneins are low molecular weight, cysteine rich peptides which bind heavy metals [1]. Their function has been linked to protection against high concentrations of heavy metals [2], metal homeostasis [3] and protection against oxidative stress [4]. While eukaryotic metallothioneins feature cysteines for metal coordination [5], first bacterial metallothioneins showed evidence for histidine residues playing an important role in metal coordination [6]. The bacterial metallothionein described best, SmtA from the cyanobacterium *Synechococcus elongatus* PCC 7942, consists of 56 amino acids and contains nine cysteine and three histidine residues. Two histidine residues are involved in metal coordination by improving protein folding and metal binding dynamics [7]. One of these molecules is able to detoxify four zinc ions [8]. Apart from the cysteine containing metallothioneins, small histidine rich peptides have been implied in metal metabolism for bacteria [9] and plants [10], being described as metallohistins.

The small size of these peptides in combination with a high content of cysteine and/or histidine made it possible to predict candidate peptides for metallothioneins and metallohistins from proteins annotated in sequenced genomes. A copper binding peptide in *Mycobacterium tuberculosis* without sequence similarity to known metallothioneins has been described [11]. In actinobacteria including the mycobacteria, further metallothioneins have not been identified although many strains have been analyzed in detail because of their potential to produce natural compounds of pharmacological interest. Even in the genome of *Mycobacterium tuberculosis,* the metallothionein had escaped identification for a prolonged period of time. Robinson [12] therefore stated that it is feasible that bacterial metal-

Correspondence: Prof. Dr. Erika Kothe, Mikrobielle Phytopathologie, Institut für Mikrobiologie, Biologisch-Pharmazeutische Fakultät, Friedrich-Schiller-Universität, Neugasse 25, D-07743 Jena, Germany
E-mail: Erika.Kothe@uni-jena.de
Phone: +49 3641 949291
Fax: +49 3641 949292
Internet: www.uni-jena.de/mikrobiologie

lothioneins are widespread, but largely overlooked in bacteria.

Here, we use a parameter-based *in silico* approach to identify putative metal binding peptides in actinobacteria, drawing on the number of sequenced bacterial genomes. Because of the characteristics of bacterial metallothioneins with regard to their size and contents of cysteines and histidines, we aimed at predicting these peptides by an *in silico* analysis.

Materials and methods

In a first step, we calculated the total amount of peptides with high concentrations of cysteine and histidine, verifying that the screening returned a feasible number of potentially metal binding peptides. The chromosomes of all 73 actinobacterial strains sequenced by Dec 10, 2009, available in public databases (Table 1) were then used to analyze peptides of up to 100 amino acids (aa) for cysteine and histidine contents. A cut-off of 15% for the lowest cysteine and histidine content qualifying for a putative metallothionein was chosen in accordance with known bacterial metallothioneins [1]. Peptides with a histidine content of ≥25% were considered as metallohistins. From this data set, similarities to known metal binding proteins were checked by BLAST searches, and conservation of cysteine and histidine residues was checked with special emphasis on known metal chelating motifs, including CXXC, CXXH, HXXC, CXC or CXH [13–17] using Vector *NTI Advance*™ 9.0 (Invitrogen™) for alignments. The identified proteins were analyzed for clustering using freely avail-

Table 1. List of completely sequenced actinobacteria used in this study.

Accession	Organism	Accession	Organism
NC_013124	*Acidomicrobium ferrooxidans* DSM 10331	NC_008769	*Mycobacterium bovis* BCG str. Pasteur 1173P2
NC_008578	*Acidothermus cellulolyticus* 11B	NC_012207	*Mycobacterium bovis* BCG str. Tokyo 172
NC_013093	*Actinosynnema mirum* DSM 43827	NC_009338	*Mycobacterium gilvum* PYR-GCK
NC_008711	*Arthrobacter aurescens* TC1	NC_011896	*Mycobacterium leprae* Br4923
NC_011886	*Arthrobacter chlorophenolicus* A6	NC_002677	*Mycobacterium leprae* TN
NC_008541	*Arthrobacter* sp. FB24	NC_010612	*Mycobacterium marinum* M
NC_013203	*Atopobium parvulum* DSM 20469	NC_008596	*Mycobacterium smegmatis* str. MC2 155
NC_012669	*Beutenbergia cavernae* DSM 12333	NC_009077	*Mycobacterium* sp. JLS
NC_008618	*Bifidobacterium adolescentis* ATCC 15703	NC_008705	*Mycobacterium* sp. KMS
NC_011835	*Bifidobacterium animalis* subsp. *lactis* AD011	NC_008146	*Mycobacterium* sp. MCS
NC_010816	*Bifidobacterium longum* DJO10A	NC_002755	*Mycobacterium tuberculosis* CDC1551
NC_004307	*Bifidobacterium longum* NCC2705	NC_009565	*Mycobacterium tuberculosis* F11
NC_011593	*Bifidobacterium longum* subsp. *infantis* ATCC 15697	NC_009525	*Mycobacterium tuberculosis* H37Ra
NC_009480	*Clavibacter michiganensis*	NC_000962	*Mycobacterium tuberculosis* H37Rv
NC_010407	*Clavibacter michiganensis* subsp. *sepedonicus*	NC_008611	*Mycobacterium ulcerans* Agy99
NC_002935	*Corynebacterium diphtheriae* NCTC 13129	NC_008726	*Mycobacterium vanbaalenii* PYR-1
NC_004369	*Corynebacterium efficiens* YS-314	NC_013235	*Nakamurella multipartita* DSM 44233
NC_006958	*Corynebacterium glutamicum* ATCC 13032	NC_006361	*Nocardia farcinica* IFM 10152
NC_009342	*Corynebacterium glutamicum* R	NC_008699	*Nocardioides* sp. JS614
NC_007164	*Corynebacterium jeikeium* K411	NC_006085	*Propionibacterium acnes* KPA171202
NC_012704	*Corynebacterium kroppenstedtii* DSM 44385	NC_010168	*Renibacterium salmoninarum* ATCC 33209
NC_010545	*Corynebacterium urealyticum* DSM 7109	NC_012490	*Rhodococcus erythropolis* PR4
NC_013170	*Cryptobacterium curtum* DSM 15641	NC_012522	*Rhodococcus opacus* B4
NC_013204	*Eggerthella lenta* DSM 2243	NC_008268	*Rhodococcus jostii* RHA1
NC_008278	*Frankia alni* ACN14a	NC_008148	*Rubrobacter xylanophilus* DSM 9941
NC_007777	*Frankia* sp. CcI3	NC_013159	*Saccharomonospora viridis* DSM 43017
NC_009921	*Frankia* sp. EAN1pec	NC_009142	*Saccharopolyspora erythraea* NRRL 2338
NC_013174	*Jonesia denitrificans* DSM 20603	NC_009953	*Salinispora arenicola* CNS-205
NC_009664	*Kineococcus radiotolerans* SRS30216	NC_009380	*Salinispora tropica* CNB-440
NC_010617	*Kocuria rhizophila* DC2201	NC_013165	*Slackia heliotrinireducens* DSM 20476
NC_013169	*Kytococcus sedentarius* DSM 20547	NC_003155	*Streptomyces avermitilis* MA-4680
NC_006087	*Leifsonia xyli* subsp. *xyli* str. CTCB07	NC_003888	*Streptomyces coelicolor* A3(2)
NC_012803	*Micrococcus luteus* NCTC 2665	NC_010572	*Streptomyces griseus* subsp. *griseus* NBRC 13350
NC_010397	*Mycobacterium abscessus*	NC_007333	*Thermobifida fusca* YX
NC_008595	*Mycobacterium avium* 104	NC_004572	*Tropheryma whipplei* str. Twist
NC_002944	*Mycobacterium avium* subsp. *paratuberculosis* K-10 NC	NC_004551	*Tropheryma whipplei* TW08 27
NC_002945	*Mycobacterium bovis* AF2122/97		

able soft-ware (MAFFT 6: http://align.bmr. kyushu-u.ac.jp/mafft/online/server, Treefinder: http://www.treefinder.de and FigTree: http://tree.bio.ed.ac.uk/software/figtree).

Because of the fact that bacterial genes are often organized in clusters or in polycistronic operons [18, 19] the genomic region surrounding the predicted metallothionein or metallohistin were checked for heavy metal related clusters or operons.

Results

Identification of putative metallothioneins and metallohistins

Peptides with high amounts of cysteine and/or histidine are strongly limited in number. Out of 285,378 proteins and peptides analyzed, 16 peptides contain 20% or more cysteine and histidine residues and only 6 peptides contain 30% or more of cysteine and histidine (Fig. 1). The peptide with the highest content of histidine was found in *Mycobacterium marinum* M with >35% histidine, lack of cysteines and a length of 68 amino acids (gi:183984821). The peptide with the highest content of cysteine was found in *Actinosynnema mirum* DSM 43827 with 19.23% (gi:256379605).

Of the entire set, 103 peptides from 49 actinobacteria were identified as potential metallothioneins and metallohistins using the search criteria size (≤100 amino acids) and cysteine and histidine content (≥15%). Out of these, 45 peptides showed similarities to known metal binding proteins or domains (Fig. 2). In addition, two peptides with an extremely high histidine content without similarities to known proteins were selected for further analysis. In the genomes of 24 strains, no potential metal binding peptides were identified by these criteria.

All putative metallothioneins and metallohistins are listed in the supplementary material.

Putative metallohistins

Eight peptides containing ≥25% histidine were identified as putative metallohistins. Four histidine rich peptides group together with two peptides from *Nocardia farcinica* IFM 10152 and *Saccharopolyspora erythraea* NRRL 2338 which appear to be derived from duplications (Fig. 3).

In proteins of *Janibacter* sp. HTCC2649 (gi:84496820) and *Nocardioides sp*. JS614 (gi:119716167), the histidine rich regions highly homologues to the predicted metallohistins are C-terminal parts of putative heavy metal efflux transporters (Fig. S1). The peptide of *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 382 is located downstream of a transcriptional regulator of the ArsR family [20], while that from *N. farcinica* IFM 10152 is located next to a gene of the TetR family [21].

In a second group, two proteins, one of *Mycobacterium marinum* M with 35.82% histidines and the other of *Mycobacterium avium* 104 annotated as a zinc transporter, Slc39a7, are clustering (Fig. 4). We could not detect similarities to metal binding regions. In addition to the

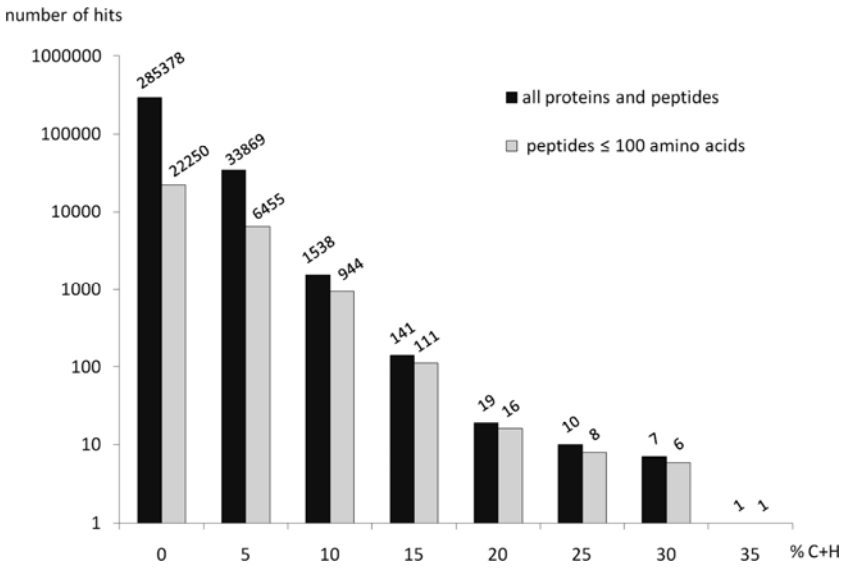

Figure 1. Relative abundance of proteins detected from annotations in sequenced genomes with high cysteine (C) and histidine (H) content. Compared are all proteins (dark bars) and peptides of a size up to 100 amino acids (light bars) for identification of potential metallothioneins and metallohistins.

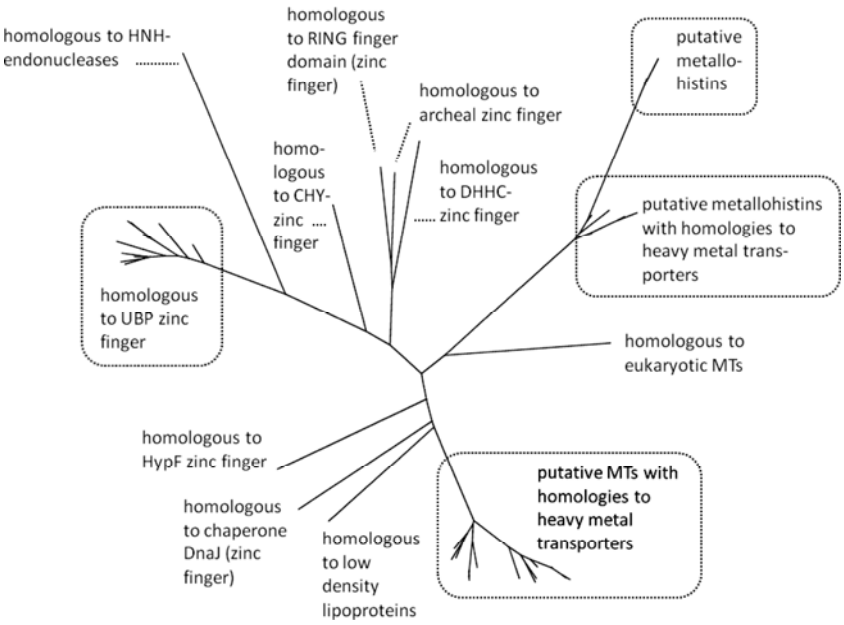

Figure 2. Rootless tree comparing identified metallohistins and metallothioneins. The clusters for the different peptides show similarities to known metal binding regions of proteins.

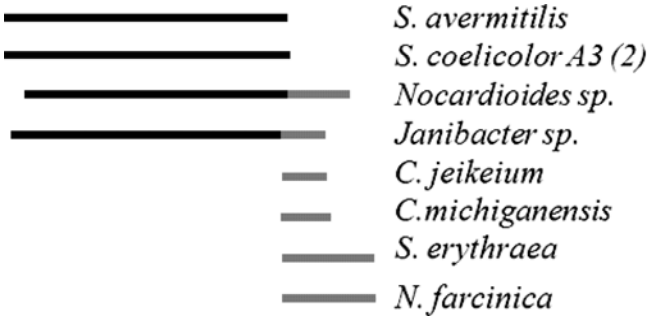

Figure 3. Evolutionary origin of metallohistins from histidine rich domains of transporter proteins. The two homologous transporters (black) from *Streptomyces avermitilis* and *S. coelicolor* do not possess the histidine rich fragment, while C-termini with high histidine content (grey) are found for transporters of *Nocardioides* sp. and *Janibacter* sp. These C-termini are found as separate peptides in *Corynebacterium jeikeium* and *Clavibacter michiganensis* and as tandem duplications with *Saccharopolyspora erythraea and Nocardia farcinica*.

high content of histidines, genes for both peptides are located downstream of genes for ArsR transcriptional regulators.

Putative metallothioneins

Among potential metallothioneins, four groups were identified: similarities were seen to zinc finger domains, heavy metal transporters, eukaryotic metallothioneins and miscellaneous. In many cases, additional metal binding motifs were noticed in the identified peptides aside of those regions which bear a similarity to known metal binding sites.

One class of peptides with similarity to zinc finger proteins contains peptides similar to zinc finger domains of the UBP type (Fig. S2). An example for such a peptide is the one from *Streptomyces avermitilis* MA-4680 with a cysteine and histidine content of 19.77% (gi:29829553). The regions preceding the zinc finger domains also contain CXH and CXXC motifs.

A peptide from *Corynebacterium glutamicum* ATCC 13032 (gi:62388969) consists of a zinc finger domain of the CHY type, referring to the N-terminal CXHY motif. In comparison to a similar protein from eukaryotic *Neurospora crassa* OR74A, three additional histidine residues are found (Fig. S3).

```
                1                                                   50
M. marinum M   (1) MSDEHTHHGPHAHEHRHGEVTHSHAHTTHQHEHVEHAHPHSHDDGTEHTH
M. avium 104   (1) MSEQHTHDAPHTHEHQHGGVTHAHAHTTHEHDHVEHEHSHAHTDGTEHTH
Consensus      (1) MS  HTH  PH HEH HG VTH HAHTTH H HVEH H H H DGTEHTH
                   51              67
M. marinum M   (51) QHVHESGLESVHSHAHS
M. avium 104   (51) RHVHQAGLEDVHQHAHA
Consensus      (51)  HVH  GLE VH HAH
```

Figure 4. Alignment of two histidine rich proteins, (gi:183984821, *Mycobacterium marinum* M, 68 aa; gi:118464477, *Mycobacterium avium* 104, 67 aa) representing potential metallohistins in bacteria.

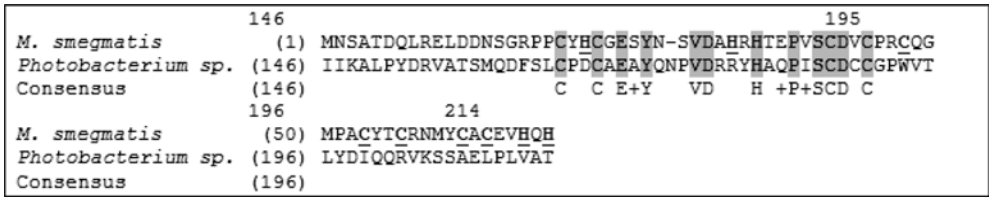

Figure 5. Alignment of a putative metallothionein (gi:118467363, *Mycobacterium smegmatis* str. MC2 155, 68 aa) and a similar zinc finger region of the HypF type (gi:89074830, *Photobacterium* sp. SKA34, 796 aa). Additional cysteines and histidines in the putative metallothionein are underlined.

A similarity to a zinc finger region of the HypF type of the hydrogenase maturation protein from *Photobacterium* sp. SKA34 was identified in *Mycobacterium smegmatis* str. MC2 155 (Fig. 5). This peptide is annotated as recombination activating protein 1. However, the similarity is restricted to the HypF zinc finger domain with four cysteines, typical for this family of zinc fingers, and two histidines being conserved. Additionally, the peptide is very rich in cysteines and histidines and contains further motifs that can be envisioned to bind metals.

A peptide from *Actinosynnema mirum* DSM 43827 (gi:256379605) shows similarity to a RING finger domain from *Paramecium tetraurelia* d4-2 (Fig. S4). The peptide of *A. mirum* DSM 43827 contains ten cysteines, representing 19.23% which is unique in available genomes of actinobacteria. Interestingly, the peptide does not contain any histidines also seen for a second peptide from *A. mirum* DSM 43827 (gi:256374337).

A peptide from *Jonesia denitrificans* (gi:256833511) shows sequence similarity to a part of a DHHC zinc finger domain (Fig. S5). Among the conserved domains are two CXXC motifs and two histidines. An additional CXXC motif is added at the C-terminal part of the peptide.

In addition to bacterial and eukaryotic zinc finger proteins, archaea have been shown to possess such metal binding proteins. A peptide from *Nakamurella multipartita* DSM 44233 (gi:258653825) shows similarity to an archaeal peptide annotated as a zinc finger protein (Fig. S6) with all cysteines conserved. Four histidines are found in addition to the cysteines in this peptide.

A peptide from *A. mirum* DSM 43827 (gi:256374337) shows similarity to the zinc finger domain of the chaperone DnaJ (Fig. S7), a protein that contains a zinc finger domain belonging to the zinc ribbon folding group [22]. Typical for this zinc binding region are four repeats of the motif CXXCXGXG. This repeat is found once in the peptide from *A. mirum* DSM 43827. This indicates that, despite similarity, it is not a zinc finger protein of this type. With one exception, the cysteine residues are conserved well, indicative of their importance for metal binding.

In a second group, we found 19 peptides with sequence similarity to N-terminal regions of heavy metal translocating P-type ATPases, for example from *Geobacter* sp. FRC-32 (gi:222053980). In the putative metallothionein from *N. farcinica* IFM 10152 (gi:54024697), several histidines appear in addition to the conserved cysteines (Fig. S8). The region with similarity to the heavy metal transporter is preceded by a region with additional three cysteines and one histidine.

One peptide from *Streptomyces avermitilis* MA-4680 contains a cysteine rich fragment with the amino acid sequence "CGCGSGCGCG" (Fig. S9). This region is exactly conserved in a metallothionein PjMT1 from *Prosopis juliflora* (gi:185178050) a heavy metal accumulating plant species [16], where the metallothionein plays a role in the binding of heavy metals. In clostridia, this region is also strongly conserved and occurs in peptides that resemble eukaryotic metallothioneins with regard to size and cysteine content, for example in *Clostridium bolteae* ATCC BAA-613 (gi:160937039).

The fourth group of putative metallothioneins with similarity to other metal binding regions of proteins is represented by two peptides belonging to two different actinobacterial species. A histidine rich peptide from *Frankia alni* ACN14a contains a sequence completely identical to the C-terminus of a HNH endonuclease from the same organism, with only a methionine added at the translation start (Fig. S10). The conserved histidines, responsible for nickel coordination in endonucleases, are present. Moreover, in comparison to colicin E9 [23], the HNH motif contains three more cysteines and four more histidines. Thus, duplication of a metal binding part of a protein seems to have occurred leading to this putative metallothionein.

A peptide found in *Sa. erythraea* NRRL 2338 shows a high cysteine and histidine content of over 21% and is unique in bacteria. A eukaryotic, cysteine rich region of low density lipoprotein-related protein 1 precursor was identified by a similarity search (Fig. S11). In the similar regions, several calcium binding sites can be found. The eukaryotic protein has a lower histidine content with 15 histidine residues having been added to a conserved

cysteine pattern, improving potential metal binding. The gene is located next to a gene encoding an iron/zinc containing superoxide dismutase, an enzyme that plays a central role in heavy metal tolerance [24, 25].

Putative metallothioneins without similarities to known metal binding regions were identified for 56 peptides (Table S14). However, since metallothioneins do not necessarily share similarities, a high cysteine and histidine content, as well as the occurrence of potential metal binding motifs like a CXXC motif, could indicate a function as a metallothionein. Thus, these peptides were added to the list of potential metallothioneins. Four of the corresponding genes were located close to genes encoding proteins which could play a role in heavy metal resistance: the gene of a peptide from *Nocardioides* sp. JS614 (gi:119717147) is located next to a copper translocating P-type ATPase, the gene of a peptide from *Arthrobacter chlorophenolicus* A6 (gi:220913762) is located close to a chromate transporter, only separated by one gene, and the genes of two peptides from *Rhodococcus opacus* B4 (gi:226362861) and *Rhodococcus* sp. RHA1 (gi:111020618) are located in the direct vicinity of genes for regulators of the TetR family.

Discussion

Metallothioneins and metallohistins are small metal binding peptides which play a central role in the resistance to high concentrations of heavy metals [2] and in the homeostasis of metals [3]. So far, only few metallothioneins have been detected in bacteria. However, the detection of a copper binding metallothionein in *Mycobacterium tuberculosis* with little sequence similarity to known metallothioneins led to the assumption that metallothioneins could be widespread in bacteria [12]. Because of the fact that metallothioneins are defined by small size and high contents of cysteine and histidine, it was possible to screen sequenced genomes for putative metallothioneins.

In this paper we predict candidate metallothioneins and metallohistins in actinobacteria by way of an *in silico* approach. We could show that the number of peptides which could function as metallothioneins or metallohistins is strongly limited. Several of the peptides identified show similarities to zinc finger domains, heavy metal transporters, eukaryotic metallothioneins and other metal binding proteins. In the predicted metallothioneins, cysteines are generally well conserved with histidines added to cysteine containing motifs and often further potential metal binding motifs are added. This indicates an optimization for the binding of heavy metals in evolution.

Zinc finger domains [26] play an essential role in establishing the three dimensional structures of proteins, for example in transcriptional regulators [27]. A range of putative metallothioneins did show similarity to zinc finger domains leading to the idea of metallothioneins being evolutionary derived from zinc finger structures.

For example, zinc finger-related peptides, containing an UBP type zinc finger, were found for multiple entries. Although bacteria contain an ubiquitin-like protein [28], ubiquitin itself does only occur in eukaryotes. Indeed, in contrast to metal binding amino acids, ubiquitin binding amino acids [29] cannot be found in the bacterial peptides indicating a change in function. The very high cysteine and histidine content, combined with the small size and a range of potential metal binding motifs, makes them candidates for potential metallothioneins.

A peptide with similarity to zinc fingers of the CHY type shows additional, albeit low similarity to a small zinc binding mitochondrial helper protein, Hot13p [30], which mediates the assembly of protein complexes. This might also be seen as an indication for functions other than metallothionein metal binding, although the enrichment for cysteine and histidine still would enable function as metallothionein.

Similarity to the zinc finger domain of HypF, was found for a peptide from *M. smegmatis* str. MC2 155, annotated as recombination activating protein 1. However, these proteins have so far been identified solely in eukaryotes [31].

Next to zinc finger domains heavy metal transporters often contain metal binding regions. In our analysis we found two groups of putative metal binding peptides with similarities.

19 putative metallothioneins show similarity to heavy metal translocating P-type ATPases featuring metal binding loops at their N-termini [32, 33].

In addition, we were able to identify peptides with strong homology to histidine rich C-termini of heavy metal transporters in *Nocardioides* sp. and *Janibacter* sp. (see Fig. 3). In *S. coelicolor* A3(2) and *S. avermitilis* MA-4680, homologous transporters do not possess the histidine rich termini. Thus, it is assumed that metal binding is not necessary for the function of these transporters but may improve performance, like with NreB from *Achromobacter xylosoxidans* [34]. The localization of genes of two of these predicted metal binding peptides next to transcriptional regulators of the ArsR and TetR family is seen to hint at a possible involvement in heavy metal regulation [35–37]. The same can be seen with

two histidine rich peptides without cysteine residues from *M. marinum* M and *M. avium* 104. These genes are both located next to transcriptional regulators of the ArsR family. The same association to a regulatory protein is seen with the metallothionein *smtA* from *Synechococcus elongatus* PCC 7942 which is located next to *smtB*, a gene for a zinc-responsive transcriptional regulator of the ArsR family. It has been shown that SmtB represses the transcription of *smtA* in the absence of heavy metals [38]. These metallohistins, lacking cysteines for metal chelation, have, to the best of our knowledge, not yet been detected in bacteria. The annotation of the histidine rich peptide from *M. avium* 104 as a zinc transporter Slc39a7 seems unwarranted, because Slc39a7 appears to be a member of the solute carrier family, the HKE4 protein from *Homo sapiens* [39, 40]. Possibly, the annotation was interpreting the high histidine content from zinc transporters of this family containing a histidine rich loop as indication of homology [41].

One peptide from *S. avermitilis* MA-4680 (gi:29830518) even shows similarity to a metal binding region from a eukaryotic metallothionein, i.e. from *P. juliflora* [16]. They share a cysteine rich fragment which also occurs in extremely cysteine rich proteins from some *Clostridium* species, so far without known function.

In summary, we were able to show that it is possible to predict promising candidates for metallothioneins and metallohistins *in silico*. Strains without encoded metallothioneins and metallohistins can also be identified fast. We found several indications of genome mutations, like duplication, release of metal binding domains being separated and now expressed in addition to the former fusion protein, or even expressed exclusively. Additionally, indications for heterologous gene transfer were traced.

Putative metallothioneins of different evolutionary origins can be shown, supporting the idea of underestimated metallothioneins in bacteria. The main function of a metallothionein/metallohistin is to sequester metals intracellularly in order to protect the cell from toxic effects of metals while storing sufficient metal to allow for the essential functions. Metal binding domains like zinc fingers have seemingly been adapted to this function during evolution.

All in all, we could show a proof-of-principle for identification of putative, small, metal binding peptides using the clade of actinobacteria. In general, this approach should also be applicable to other bacterial or archaeal groups.

Acknowledgements

We want to thank Dr. Matthias Gube for his introduction to DNA based search tools and Naomi and Rebecca Thompson for correcting the English. The DFG funded Gk1257 is thanked for support.

References

[1] Robinson, N.J., Whitehall, S.K., Cavet, J.S., 2001. Microbial metallothioneins. Adv. Microb. Physiol., **44**, 183–213.

[2] Blindauer, C.A., Harrison, M.D., Parkinson, J.A., Robinson, A.K., Cavet, J.S., Robinson, N.J., Sadler, P.J., 2001. A metallothionein containing a zinc finger within a four-metal cluster protects a bacterium from zinc toxicity. Proc. Natl. Acad. Sci. USA., **98**, 9593–9598.

[3] Suhy, D.A., Simon, K.D., Linzer, D.I., O'Halloran, T.V., 1999. Metallothionein is part of a zinc-scavenging mechanism for cell survival under conditions of extreme zinc deprivation. J. Biol. Chem., **274**, 9183–9192.

[4] Palmiter, R.D., 1998. The elusive function of metallothioneins. Proc. Natl. Acad. Sci. USA, **95**, 8428–8430.

[5] Piccinni, E., Bertaggia, D., Santovito, G., Miceli, C., Kraev, A., 1999. Cadmium metallothionein gene of *Tetrahymena pyriformis*. Gene, **234**, 51–59.

[6] Blindauer, C.A., Sadler, P.J., 2005. How to hide zinc in a small protein. Acc. Chem. Res., **38**, 62–69.

[7] Blindauer, C.A., Razi, M.T., Campopiano, D.J., Sadler, P.J., 2007. Histidine ligands in bacterial metallothionein enhance cluster stability. J. Biol. Inorg. Chem., **12**, 393–405.

[8] Blindauer, C.A., Harrison, M.D., Robinson, A.K., Parkinson, J.A., Bowness, P.W., 2002. Multiple bacteria encode metallothioneins and SmtA-like zinc fingers. Mol. Microbiol., **45**, 1421–1432.

[9] Gilbert, J.V., Ramakrishna, J., Sunderman, F.W. Jr., Wright, A., Plaut, A.G., 1995. Protein Hpn: cloning and characterization of a histidine rich metal binding polypeptide in *Helicobacter pylori* and *Helicobacter mustelae*. Infect. Immun., **63**, 2682–2688.

[10] Gupta, R.K., Dobritsa, S.V., Stiles, C.A., Essington, M.E., Liu, Z. *et al.*, 2002. Metallohistins: a new class of plant metal binding proteins. J. Protein. Chem., **21**, 529–536.

[11] Gold, B., Deng, H., Bryk, R., Vargas, D., Eliezer, D. *et al.*, 2008. Identification of a copper-binding metallothionein in pathogenic mycobacteria. Nat. Chem. Biol., **4**, 609–616.

[12] Robinson, N.J., 2008. A bacterial copper metallothionein. Nat. Chem. Biol., **4**, 582–583.

[13] Zhou, L., Singleton, C., Le Brun, N.E., 2008. High Cu(I) and low proton affinities of the CXXC motif of *Bacillus subtilis* CopZ. Biochem. J., **413**, 459–465.

[14] Entus, R., Poling, M., Herrmann, K.M., 2002. Redox regulation of *Arabidopsis* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. Plant Physiol., **129**, 1866–1871.

[15] Wang, J., Luthey-Schulten, Z.A., Suslick, K.S., 2003. Is the olfactory receptor a metalloprotein? Proc. Natl. Acad. Sci. USA, **100**, 3035–3039.

[16] Usha, B., Venkataraman, G., Parida, A., 2009. Heavy metal and abiotic stress inducible metallothionein isoforms from *Prosopis juliflora* (SW) D.C. show differences in binding to heavy metals *in vitro*. Mol. Genet. Genomics, **281**, 99–108.

[17] Van Horn, J.D., Bulaj, G., Goldenberg, D.P., Burrows, C.J., 2003. The Cys-Xaa-His metal binding motif: [N] versus [S] coordination and nickel-mediated formation of cysteinyl sulfinic acid. J. Biol. Inorg. Chem., **8**, 601–610.

[18] Xie, P., Zeng, A., Qin, Z., 2009. *CmdABCDEF*, a cluster of genes encoding membrane proteins for differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). BMC Microbiol., **9**, 157.

[19] Misra, T.K., 1992. Bacterial resistances to inorganic mercury salts and organomercurials. Plasmid, **27**, 4–16.

[20] Busenlehner, L.S., Pennella, M.A., Giedroc, D.P., 2003. The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. FEMS Microbiol. Rev., **27**, 131–143.

[21] Ramos, J.L., Martínez-Bueno, M., Molina-Henares, A.J., Terán, W., Watanabe, K., 2005. The TetR family of transcriptional repressors. Microbiol. Mol. Biol. Rev., **69**, 326–356.

[22] Krishna, S.S., Majumdar, I., Grishin, N.V., 2003. Structural classification of zinc fingers: survey and summary. Nucleic Acids Res., **31**, 532–550.

[23] Kleanthous, C., Kühlmann, U.C., Pommer, A.J., Ferguson, N., Radford, S.E. *et al.*, 1999. Structural and mechanistic basis of immunity toward endonuclease colicins. Nat. Struct. Biol., **6**, 243–252.

[24] Fridovich, I., 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem., **64**, 97–112.

[25] Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Radic. Biol. Med., **18**, 321–336.

[26] Klug, A., Schwabe, J.W., 1995. Protein motifs 5. Zinc fingers. FASEB J., **9**, 597–604.

[27] Bouhouche, N., Syvanen, M., Kado, C.I., 2000. The origin of prokaryotic C_2H_2 zinc finger regulators. Trends Microbiol., **8**, 77–81.

[28] Pearce, M.J., Mintseris, J., Ferreyra, J., Gygi, S.P., Darwin, K.H., 2008. Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis*. Science, **322**, 1104–1107.

[29] Reyes-Turcu, F.E., Horton, J.R., Mullaly, J.E., Heroux, A., Cheng, X. *et al.*, 2006. The ubiquitin binding domain ZnF UBP recognizes the C-terminal diglycine motif of unanchored ubiquitin. Cell, **124**, 1197–208.

[30] Curran, S.P., Leuenberger, D., Leverich, E.P., Hwang, D.K., Beverly, K.N. *et al.*, 2004. The role of Hot13p and redox chemistry in the mitochondrial TIM22 import pathway. J. Biol. Chem., **279**, 43744–43751.

[31] De, P., Rodgers, K.K., 2004. Putting the pieces together: identification and characterization of structural domains in the V(D)J recombination protein RAG1. Immunol Rev., **200**, 70–82.

[32] Banci, L., Bertini, I., Ciofi-Baffoni, S., Su, X.C., Miras, R., Bal, N., 2006. Structural basis for metal binding specificity: the N-terminal cadmium binding domain of the P1-type ATPase CadA. J. Mol. Biol., **356**, 638–650.

[33] Nies, D.H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol. Rev., **27**, 313–339.

[34] Grass, G., Fan, B., Rosen, B.P., Lemke, K., Schlegel, H.G. *et al.*, 2001. NreB from *Achromobacter xylosoxidans* 31A Is a nickel-induced transporter conferring nickel resistance. J. Bacteriol., **183**, 2803–2807.

[35] Kar, S.R., Adams, A.C., Lebowitz, J., Taylor, K.B., Hall, L.M., 1997. The cyanobacterial repressor SmtB is predominantly a dimer and binds two Zn2+ ions per subunit. Biochemistry, **36**, 15343–15348.

[36] Van Zile, M.L., Cosper, N.J., Scott, R.A., Giedroc, D.P., 2000. The zinc metalloregulatory protein *Synechococcus* PCC7942 SmtB binds a single zinc ion per monomer with high affinity in tetrahedral coordination geometry. Biochemistry, **39**, 11818–11829.

[37] Kloosterman, T.G., van der Kooi-Pol, M.M., Bijlsma, J.J., Kuipers, O.P., 2007. The novel transcriptional regulator SczA mediates protection against Zn2+ stress by activation of the Zn2+-resistance gene *czcD* in *Streptococcus pneumoniae*. Mol. Microbiol., **65**, 1049–1063.

[38] Cook, W.J., Kar, S.R., Taylor, K.B., Hall, L.M., 1998. Crystal structure of the cyanobacterial metallothionein repressor SmtB: a model for metalloregulatory proteins. J. Mol. Biol., **275**, 337–346.

[39] Taylor, K.M., Morgan, H.E., Johnson, A., Nicholson, R.I., 2004. Structure-function analysis of HKE4, a member of the new LIV-1 subfamily of zinc transporters. Biochem. J., **377**, 131–139.

[40] Gaither, L.A., Eide, D.J., 2001. Eukaryotic zinc transporters and their regulation. Biometals, **14**, 251–270.

[41] Huang, L., Kirschke, C.P., Zhang, Y., Yu, Y.Y., 2005. The ZIP7 gene (*Slc39a7*) encodes a zinc transporter involved in zinc homeostasis of the Golgi apparatus. J. Biol. Chem., **280**, 15456–15463.

3.6 Streptomycete heavy metal resistance: Extracellular and intracellular mechanisms

Erika Kothe, Christian Dimkpa, Götz Haferburg, André Schmidt, Astrid Schmidt and Eileen Schütze

In: Sherameti. I., Varma, A. (eds.), Soil Heavy Metals, Kapitel 10, Verlag Springer Berlin Heidelberg, ISBN 978-3-642-02436-8

Chapter 10
Streptomycete Heavy Metal Resistance: Extracellular and Intracellular Mechanisms

Erika Kothe, Christian Dimkpa, Götz Haferburg, André Schmidt, Astrid Schmidt, and Eileen Schütze

10.1 Introduction

Heavy metals in the environment influence microbial populations (Haferburg and Kothe 2007). These heavy metals mainly derive from mining activities; 240,000 km^2 of the Earth's surface are influenced by mining (Furrer et al. 2002). However, there are also some environments where high metal loads have arisen due to geogenic sources, such as serpentinite soils that have developed on nickel-rich rock substrates. Plants that are able to accumulate high nickel levels have been described, especially on serpetinites, and microbial populations that are resistant to high nickel concentrations have been found in the rhizospheres of such hyperaccumulator plants and characterized (Mengoni et al. 2001; Park et al. 2004; Schlegel et al. 1991). The evolution of metal resistance can therefore be traced back to habitats that are geogenic; the subsequent spread of microorganisms or the heterologous transfer of microbial resistance mechanisms to anthropogenic metal-rich niches can thus be envisioned.

In mining areas, heavy metal loads can reach extreme levels. This is mainly due to acid mine or rock drainage (AMD, ARD). During this process, the acidification of the environment leads to high heavy metal solubility (Singer and Stumm 1970). The AMD process is accelerated by microbial processes in which the oxidation of iron leads to the release of protons and acidification (Collins and Stotzky 1992). The exposure of sulfide ores to environmental conditions and oxygen in the air allows bacteria like *Acidithiobacillus ferrooxidans* or *Leptosprillum* species to gain energy from the oxidation of iron or manganese (Schippers et al. 1996). This leads to acidification, and the oxidation power keeps the reaction running for as long as

E. Kothe (✉), C. Dimkpa, G. Haferburg, A. Schmidt, A. Schmidt, and E. Schütze
Institute for Microbiology, Friedrich-Schiller- University Jena,
Neugasse 25, 07743, Jena, Germany
e-mail: Erika.kothe@uni-jena.de
e-mail: C.Dimkpa@uni-jena.de
e-mail: gotz.haferburg@uni-jena.de
e-mail: smiddy74@yahoo.de
e-mail: smiddy74@yahoo.de
e-mail: eileen-schuetze@web.de

I. Sherameti and A. Varma (eds.), *Soil Heavy Metals*, Soil Biology, Vol 19,
DOI 10.1007/978-3-642-02436-8_10, © Springer-Verlag Berlin Heidelberg 2010

reduced metal (usually present as a sulfide ore) and oxygen are available. This results in the extremely acidic and metal-rich seepage waters that can be found downstream from historic and recent mines and mining heaps. An extreme example would be the Rio Tinto region in Spain, where the high iron content and subsequent precipitation of iron hydroxides lead to waterways with orange to deep-red coloration and well-adapted biota (Zettler et al. 2003).

Bacteria cope with metals in their specific environments in different ways. The mechanisms of heavy metal resistance include extracellular and intracellular sequestration, lowering the concentration of bioavailable metal in the direct surroundings of the cell, or reducing the toxicity of the metal by changing its chemical oxidation state. Another resistance mechanism involves the expression of specific exporter proteins that keep the intracellular concentration of the metal at a tolerable level (for a recent review, see Haferburg and Kothe 2007). Other mechanisms involved in enhanced metal resistance help the cell to cope with the toxic effects of heavy metals. One specific mechanism is the formation of reactive oxygen species (ROS) via the Fenton reaction. In order to cope with the ROS, superoxide dismutases, catalases and peroxidases that relieve the cells of the adverse effects of ROS are expressed.

In aquatic environments with high metal loads, the metal concentrations around the cell are kept constant as metal is constantly delivered to the cell surface. The cells can counteract the toxic effects best if they express highly specific metal exporter proteins for use as an optimal safety guard. In soil, in contrast, the direct surroundings of the cell can be depleted of metals by excreting components that alter the bioavailability of the metal. In this way, the environment of the cell can be altered without constant resupplying the metal. Thus, mechanisms of extracellular sequestration are more likely to be found in soil microbes, while motile bacteria are more likely to have evolved export mechanisms. This hypothesis is supported by the discovery of highly specific metal exporter systems in Gram-negative bacteria (for review: Nies 2003), while filament-forming streptomycetes have been shown to possess various mechanisms for extracellular sequestration. In both environments, intracellular sequestration and mechanisms to prevent ROS damage will further enhance heavy metal resistance. Here, we summarize the mechanisms that together lead to very high heavy metal resistance in streptomycetes, an important group of soil bacteria.

10.2 Streptomycetes Are a Prominent Population in Heavy Metal Contaminated Soils

Streptomycetes are prominent in all soils. This is even evident from the smell of fresh soil – the volatile substances associated with fresh earth are geosmins, which are produced by streptomycetes. Streptomycetes have a very active secondary metabolism and can produce a wide variety of chemicals. Among these chemicals are antibiotics; up to 80% of the antibiotics used by man originated from

streptomycete metabolites (Haferburg et al. 2009; Hopwood 2006). These antibiotics are thought to provide the population that produces them with a competitive edge compared to other taxa in the soil.

In normal soils, streptomycetes – or more generally, actinobacteria – comprise about 20% of the bacterial population. Filamentous growth, the formation of hyphae, can be interpreted as an adaptation to living in soil, where nutrients may be unequally distributed spatially. The formation of a mycelium in which nutrients can be transported from one area to another enables growth under conditions where some of the nutrients needed by single-celled bacteria may be available in their immediate surroundings while others are not, and can also lead to a more constant water supply in an environment prone to drying out and rewetting. The same mechanism is utilized by fungi, which also form a high proportion of the soil biomass.

An additional adaptive advantage under dry conditions is the production of spores. This gives Gram-positives with endospore-forming bacilli and clostridia, as well as actinomycetes, a competitive edge when growing in the soil. Actinomycetes do not form endospores, but are able to differentiate spores from the aerial mycelium, either in spore chains (e.g., streptomycetes) or in sporangia (e.g., actinoplanetes). These spores are not as resistant to chemicals or heating as the endospores of Gram-positive bacteria with low genomic G+C contents (bacilli and clostridia), but they are nevertheless able to withstand heat and lack of moisture, which are conditions that can be found in natural soils. Upon the return of moist and temperate conditions, the spores germinate, forming new substrate mycelium for feeding and, subsequently, aerial mycelium for dispersing spores and spreading them to new environments.

These specific features of streptomycetes – the highly active secondary metabolism as well as the mycelial growth and spore production – appear to be mechanisms that also provide advantages under other adverse conditions, including heavy metal contamination of terrestrial environments. In a former uranium mining site near Ronneburg in Thuringia, Germany, the population of soil microbes was analyzed by cultivation-dependent and cultivation-independent DNA-based methods (Haferburg et al. 2007; Schmidt et al. 2005). It was found that, in contrast to uncontaminated soils from temperate regions, the population was highly enriched in Gram-positive bacteria, with bacilli and streptomycetes clearly dominating over Gram-negative proteobacteria, which often form large parts of the population in normal soil (Fig. 10.1). Thus, the advantages described above can be assumed to aid growth in poor soils contaminated with metals, as observed in the former mining district at Ronneburg.

10.3 Isolation of Heavy Metal Resistant Streptomycetes

The field site studied in the former uranium mining area at Ronneburg, Thuringia, Germany, is situated on a former heap leaching site (Kothe et al. 2005). The leaching process led to high infiltration of metal contaminants into the ground, and these

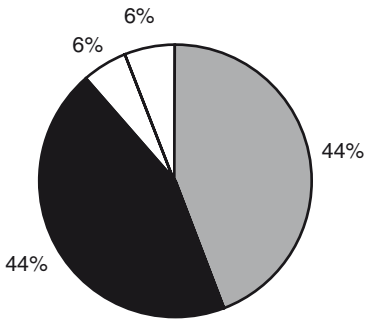

Fig. 10.1 Proportions of taxa identified in metal-contaminated soil at a former uranium mining site in Ronneburg, Germany. Actinomycetes and bacilli each comprised 44%, while both proteobacteria and non-spore-forming Gram positives with low G+C contents provided 6% of the taxa identified

contaminants are still present after removing the heap material in the 1990s (Geletneky et al. 2002). The mining activity did not start until 1949, which places an upper limit on the time available for adaptive processes to prompt the development of specialized, heavy metal resistant microbial consortia. In addition to the acidity of the leaching process, the bioavailability of heavy metals is especially high in this setting. We therefore asked whether microbial consortia that possess high heavy-metal resistance towards multiple contaminants are present in this environment. As a comparison, we chose a highly contaminated (albeit less acidic) environment in Argentina, where a ditch receiving water from a copper filter plant has operated for just 15 years in the vicinity of Tucuman City. To have a presumably uncontaminated control site, city parks at Jena, Germany were chosen. Although tolerant (defined as a tolerance of up to 0.2 mM $NiCl_2$) and even highly resistant (over 5 mM $NiCl_2$) strains were isolated from uncontaminated sites, we found only three sensitive strains. At the field site tested, 25% of the strains were resistant, while at the Argentina ditch site, as many as 50% of the strains were highly resistant to heavy metals. Thus, 15 or 50 years were sufficiently long periods to allow the establishment of a community comprising highly resistant actinobacteria.

Since highly resistant strains were isolated, we investigated the highest level of resistance exhibited by isolates obtained from the field site tested.

It is important to note that resistance levels published in the literature are hard to compare. The different media used to grow the strains make it almost impossible to compare the bioavailable fraction of heavy metals between studies, and this is the sole determinant of ecotoxicity. We used minimal media, since it has been noted that ingredients in complex media could complex metals and thus lead to artificially high estimates for the levels of resistance (Amoroso et al. 2000). In defined salt media such as those we used to grow streptomycetes, the complexation of heavy metals can only be achieved with excreted substances, and is thus linked to microbial activity. In this case, the estimates of resistance levels are more likely to reflect the actual physiological capacity of the bacteria to cope with stressors such as heavy metals in their environment.

10.4 Metal Resistance Characterization and Adaptation

In a first screening, more than 200 strains were individually tested for resistance towards different heavy metals, including nickel, cadmium, lead, mercury, zinc, copper, and chromium. Nickel was afforded special attention, since it is representative of many metals that can be found in trivalent and bivalent states in nature and is mobilized under even moderately acidic conditions. The strain with the highest resistance level was able to grow on minimal solid media containing 130 mM nickel (Schmidt et al. 2009a) This is more than 8,000 ppm – the medium appeared green due to the dissolved nickel. We tested whether this strain would be able to grow on soil agar prepared from contaminated soil (not soil extract) with 6,000 ppm nickel, and we observed clear growth on these plates, showing that strain P16-B1 has the ability to colonize the soil even under high nickel stress, even when the soil was co-contaminated with other heavy metals (Schmidt et al. 2009a). We can thus conclude that the streptomycete population at our field site had attained extremely high metal resistance levels through adaption over a limited time frame. Since it was logical to assume that such high resistance levels are only possible when different metal resistance mechanisms are exploited concomitantly, we investigated the different routes that allow microorganisms to achieve heavy metal tolerance.

10.5 Chelators and Siderophores

The bioavailability of metals can be altered by forming metal complexes with chelating molecules (or minerals, as discussed in the next section). The excretion of chelating substances has been described for bacteria and fungi, with the most active components being siderophores that are involved in iron acquisition (Crowley et al. 1984). Under oxic conditions, iron is only minimally bioavailable, and the very low solubility of Fe^{3+} hinders the uptake of iron required for electron transport chain components and in active centers of redox-active enzymes. This is especially true of aerobic, soil-dwelling organisms, including plant roots. While plants mainly acquire iron through the acidification of the rhizosphere and the excretion of organic acids such as oxalate, citrate or malate, bacteria apply another strategy. They excrete low molecular weight, high iron binding affinity substances – siderophores, which are delivered and in many cases taken up after iron loading. Many different siderophores have been described for both Gram-negative and Gram-positive bacteria. Hydroxamate siderophore production was verified in *Streptomyces acidiscabies* E13, a nickel-resistant isolate from the field test site (Dimkpa et al. 2008). The three different hydroxamate-type siderophores can be produced irrespective of the presence of nickel in the cultures, showing that their respective production mechanisms are not influenced by metals other than iron. The effects of the siderophores on the bioavailabilities of both iron and nickel were shown in plant experiments, where the addition of filtrates of hydroxamate-producing cultures of *S. acidiscabies* E13

had a plant growth promoting effect on cowpea seedlings (Dimkpa et al. 2008). The three hydroxamates found in the culture filtrates are the desferroxamines DFOE (with a circular structure) and DFOB (with an open structure), as well as coelichelin, which has an open structure with even less rigidity in the backbone. In accordance with these structural predispositions, the amount of siderophore bound to iron versus that bound to nickel varied among the three molecular types of siderophore, and with time. Similar amounts of iron and nickel were found to bind to the siderophores in the overwhelming presence of nickel, the production of all three siderophores was possible at the same time, and the production of all three siderophores changed over time (Dimkpa et al. 2008). While DFOE predominantly chelated nickel, DFOB showed a preference for iron, especially at the peak in production, while large portions of coelichelin remained unchelated, even though nickel was present at 2 mmol L^{-1} in the experiment while iron was limiting. Thus, the excretion of siderophores leads to nickel complexation in the surroundings of the bacterial hyphae, and while nickel is chelated, iron is still solubilized, aiding iron reduction and uptake into the cell. The siderophore thus plays a dual role in releasing nickel stress and allowing sufficient iron to be taken up for biosynthesis and metabolism.

Another observation made about the heavy metal resistant strains isolated was that many of them produced a dark pigment. Soluble brownish pigments are known to be formed by many streptomycetes, and it has been suggested that these melanin-like pigments are able to sequester metals from the vicinity of the growing cell. Indeed, cultures grown in media with increasing amounts of added nickel were darker, indicating the increased production of the melanin-like pigment (Fig. 10.2). In order to address these questions, mutant strains were derived from a UV-directed mutagenesis. White mutants were obtained, but did not show an unequivocally lower nickel resistance. It can thus be concluded that nickel sequestration is not driven mainly by melanin excretion, although the substance may still have a slight protective effect. Such an effect could also be due to the release of ROS stress, as melanin is also known to scavenge oxygen radicals through the

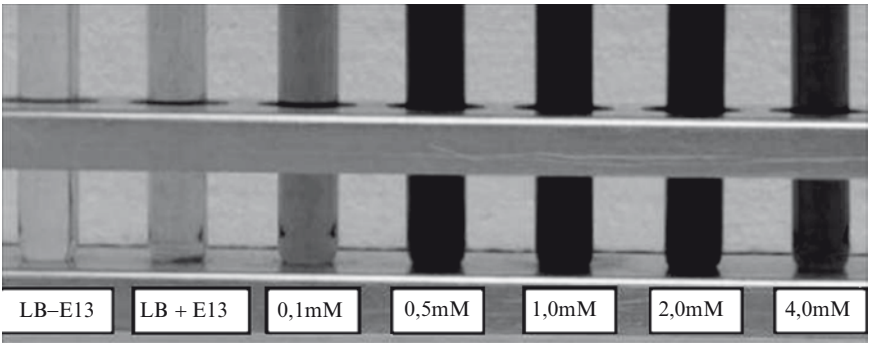

Fig. 10.2 Increased production of a melanin-like brown pigment under nickel stress by *S. acidiscabies* E13

radical-assisted formation of higher molecular weight melanins. The role of the siderophores clearly exceeded the potential function of the melanins in protecting from nickel stress.

10.6 Biomineralization

In addition to chelation, the induction of mineral formation can lead to metal depletion in the direct surroundings of a bacterial cell. Biomineralization is the process in which microorganisms aid the growth of crystals by providing either a crystallization initiator or anions for mineralization. Both of these approaches to biomineralization depend on the presence of cells, and the latter depends on the presence of actively growing, living cells. One well-studied example of biomineral formation is provided by the intracellular magnetite-containing bodies formed by magnetotactic bacteria (Bäuerlein 2003). An extracellular biomineralization process appears to be more conducive to metal resistance. The formation of hydrozincite in zinc-rich mine effluents has been described (De Giudici et al. 2007). There, the precipitates formed with bacterial inocula were the dominant species of zinc in the AMD waters. This biomineralization would also allow growth under high nickel stress. Indeed, the formation of a new biomineral with *S. acidiscabies* E13 was demonstrated (Haferburg et al. 2008). The natural mineral struvite is composed of Mg $(NH_4)(PO_4)$ $6H_2O$; nickel struvite, as we termed the biomineral observed in our cultures, is its nickel analog: $Ni(NH_4)(PO_4)$ $6H_2O$. The crystals form on top of colonies growing on solid media, but also in liquid cultures of the strain. The medium contains not ammonia but nitrate as the nitrogen source, which does not allow mineralization without active metabolism of the streptomycete. The mineral formed is extremely pure, with only minimal magnesium impurities, which is especially interesting because the natural mineral struvite could also have formed, using magnesium from the medium. However, this was not observed, which suggests a biologically controlled process. Inoculation of the media with dead biomass did not lead to nickel/struvite formation, showing that it indeed is an active, biologically controlled process that warrants the term "biomineralization." With nickel bound to a mineral phase, the cells clearly have an adaptive advantage in terms of released nickel stress.

10.7 Cell Wall Adsorption

Adsorption to extracellular surfaces also reduces the available metal concentration. In Gram-positive bacteria, the cell wall is accessible from the outside with no outer membrane protecting the surface. This leads to different binding capacities from Gram-negative bacteria. The possibility of metal binding to the cell wall or protein layers has been shown for *Bacillus sphaericus*, where attachment to S-layer

proteins allows metal sequestration (Merroun et al. 2005). This has been used in the formulation of Biocer, a ceramic with embedded *B. sphaericus* cells that is used for water treatment.

The ability of streptomycetes to bind metals to the extracellular cell wall fraction was shown by differential centrifugation. Depending on the strains used, up to 8,000 ppb nickel were found to be associated with the cell wall fraction. The strain with the highest retention capacity was *Streptomyces lincolnensis* Tosca3, while 800–1,000 ppb nickel were bound to the cell wall in *S. acidiscabies* E13, the strain used in the aforementioned studies.

In an experimental setup that was employed to show heavy metal sequestration by cells added to AMD waters, *S. acidiscabies* was shown to retain large amounts (up to 80%) of Al, Mn, Co, Ni and U, and even 90% of copper, even when dead biomass was used (Haferburg et al. 2007). This clearly indicated binding to cell wall fractions, as no active metabolism is available. In addition, rare earth elements (REEs) were retained, in the case of dead biomass, to levels of about 50%. Here, living inoculum had a greater effect, showing that metabolic activities are most likely involved in REE sequestration. The pattern of REE implied fractionation, meaning that the heavy REEs were retained better than light REEs. Such a pattern of fractionation is an indication of biological processes. In the case of the cell wall, this may be that the ionic radii of heavy REEs are better suited to binding to the cell wall components. This allows the use of the REE fractionation pattern to predict biological reactions in geohydrochemical source–sink evaluations (Merten et al. 2005).

10.8 Intracellular Storage

Potentially toxic heavy metals are usually taken up through the transporter systems that are used to acquire essential micronutrients, such as potassium for monovalent ions or calcium for bivalent ions. These low-affinity uptake systems could also take up other metals, including sodium, copper, cadmium, zinc, nickel or lead, to give just a few examples. While nickel and zinc have biological functions, only toxic effects have been reported for other metals. For the essential metals nickel, copper and zinc, intracellular homeostasis is needed to prevent damage to proteins or DNA. A highly specific efflux system could provide this homeostasis (Grass et al. 2000; for a review see Nies 2003 and references therein); nevertheless, chaperones for the metals are required to keep the intracellular stock from damaging cell components. It can therefore be assumed that metals such as nickel could be bound to chaperone molecules in the cell (Haferburg and Kothe 2007 and references therein). We could envision that phosphate, with its negative charge, could provide such a chaperone function, and that metals could be stored in volutin granules of polyphosphates. However, results from preliminary experiments indicate that a lack of polyphosphate kinase does not lead to increased sensitivity in *S. acidiscabies* E13 (Haferburg, unpublished).

In order to search for intracellular storage molecules for nickel, a fractionation by gel filtration was performed. The fractions with large amounts of nickel were of low molecular weight and contained only small amounts of protein, indicating a high binding capacity. Proteins with high contents of histidine and cysteine amino acid moieties can be detected in the genomes of the fully sequenced streptomycetes *Streptomyces coelicolor* and *Streptomyces avermitilis*. Such proteins are currently being investigated for their role in intracellular nickel storage.

One of the enzymes in streptomycetes that is known to require nickel at its active center is nickel-dependent superoxide dismutase (NiSOD). SOD is involved in ROS detoxification, making this special enzyme interesting for two reasons. Overexpression of SOD has been shown to lead to heavy metal tolerance in yeast, and the expression level of NiSOD in resistant strains was investigated to see whether NiSOD plays a protective role under in situ conditions in contaminated soil (Schmidt et al. 2007). In addition, NiSOD itself is interesting, since the enzyme is structurally different from other SODs (Schmidt et al. 2009b). The nickel-resistant strains provide a source for biochemical characterization, since nickel in the medium increases SOD expression, and so highly expressed enzymes can be purified.

Proteome analyzes have been performed to detect regulatory mechanisms induced by nickel stress (Schmidt et al. 2005). Cells grown with nickel clearly show the induction of several proteins, among them regulators of the TetR family. Analysis of the corresponding genes will allow the identification of regulatory mechanisms for cells that must cope with heavy metal stress.

10.9 Conclusion

As can be seen from the analysis of streptomycetes, bacteria can employ different mechanisms to achieve heavy metal tolerance or resistance. Especially for strains that can tolerate extreme amounts of metals, the coordinated activities of several different mechanisms of heavy metal resistance, including intracellular and extracellular sequestration and excretion of components to alter the local environment, are clearly necessary. Adaptations leading to the acquisition of several of these mechanisms may be explained by heterologous gene transfer. Indeed, most of the strains investigated contain extrachromosomal plasmids, as shown by pulse field gel electrophoresis (Schmidt et al. 2005). Whether or not these contain resistance genes, and whether or not these plasmids are from streptomycetes or elsewhere, remain to be seen. Nevertheless, it has become clear that adaptive processes, even those that occur over a limited time frame of approximately 50 years, can lead to highly resistant strains that utilize multiple resistance mechanisms, all of which contribute to heavy metal resistance in an environment influenced by AMD.

All of the above indicate that microbes could be used for bioremediation purposes (Kothe et al. 2005; Haferburg and Kothe 2007; Haferburg et al. 2007). Many of the studies investigating heavy metal resistance in microorganisms are being performed in order to find strains that are suitable for just such a purpose

(Albarracín et al. 2008; Sineriz et al. 2009). The remediation of AMD is clearly one of the major tasks to be tackled in the future (Johnson and Hallberg 2005), and in order to optimize strategies for bioremediation, a deeper knowledge of the basic biological mechanisms involved in heavy metal resistance is needed.

Acknowledgements This work was supported by IMPRS (Max-Planck Society), HIGRADE (Helmholtz Association), Gk1257 and JSMC (DFG).

References

Albarracín VH, Winik B, Kothe E, Amoroso MJ, Abate CM (2008) Copper bioaccumulation by the actinobacterium *Amycolatopsis sp.* AB0. J Basic Microbiol 48:323–330

Amoroso M-J, Schubert D, Mitscherlich P, Schumann P, Kothe E (2000) Evidence for high affinity nickel transporter genes in heavy metal resistant *Streptomyces spec*. J Basic Microbiol 40: 295–301

Bäuerlein E (2003) Biomineralization of unicellular organisms: An unusual membrane biochemistry for the production of inorganic nano- and microstructures. Ang Chem 42:614–641

Collins YE, Stotzky G (1992) Heavy metals alter the electrokinetic properties of bacteria, yeasts and clay minerals. Appl Environ Microbiol 58:1592–1600

Crowley DE, Wang YC, Reid CPP, Szanislo PJ (1984) Mechanisms for iron acquisition from siderophores by micro organisms and plants. Plant Soil 130:179–198

De Giudici G, Podda F, Caredda A, Tombolino R, Casu M, Ricci C (2007) *In vitro* investigation of hydrozincite biomineralization. Water Rock Interact 12:415–418

Dimkpa C, Svatos A, Merten D, Büchel G, Kothe E (2008) Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress. Can J Microbiol 54:163–172

Furrer G, Phillips BL, Ulrich K-U, Pöthig R, Casey WH (2002) The origin of aluminium flocs in polluted streams. Science 297:2245–2247

Geletneky J, Paul M, Merten D, Büchel G (2002) Impact of acid rock drainage in a discrete catchment area at the former uranium mining site Ronneburg (Germany). In: Nelson JD, Cincilla WA, Foulk CL, Hinshaw LL, Ketellaper V (eds) Tailings and mine waste, Proceedings ninth international conference on trainings and mine waste, Fort Colllings, CO, pp 67–74

Grass G, Grobe C, Nies DH (2000) Regulation of the cnr cobalt and nickel resistance determinant from *Ralstonia sp.* strain CH34. J Bacteriol 182:1390–1398

Haferburg G, Kothe E (2007) Microbes and metals: interactions in the environment. J Basic Microbiol 47:453–467

Haferburg G, Merten D, Büchel G, Kothe E (2007) Biosorption capacity of metal tolerant microbial isolates from a former uranium mining area and their impact on changes in rare earth element patterns in acid mine drainage. J Basic Microbiol 47:474–484

Haferburg G, Groth I, Möllmann U, Kothe E, Sattler I (2009) Arousing sleeping genes: Shifts in secondary metabolism of metal tolerant actinobacteria under conditions of heavy metal stress. Biometals 22:225–234

Haferburg G, Klöß G, Schmitz W, Kothe E (2008) "Ni-struvite"– a new biomineral formed by a nickel resistant *Streptomyces acidiscabies*. Chemosphere 72:517–523

Hopwood DA (2006) Soil to genomics: the *Streptomyces* chromosome. Annu Rev Genet 40:1–23

Johnson DB, Hallberg K (2005) Acid mine drainage remediation options: a review. Sci Total Environ 338:3–14

Kothe E, Bergmann H, Büchel G (2005) Molecular mechanisms in bio-geo-interactions. Chemie Erde 65S1:7–27

Mengoni A, Barzanti R, Gonnelli C, Gabbrielli R, Bazzicalupo M (2001) Characterization of nickel-resistant bacteria isolated from serpentine soil. Environ Microbiol 3:691–698

Merroun ML, Raff J, Rossberg A, Hennig C, Reich T, Selenska-Pobell S (2005) Complexation of uranium by cells and S-layer sheets of *Bacillus sphaericus* JG-A12. Appl Environ Microbiol 71:5532–5543

Merten D, Geletneky J, Bergmann H, Haferburg G, Kothe E, Büchel G (2005) Rare earth element patterns: a tool for remediation of acid mine drainage. Chem Erde 65S1:97–114

Nies DH (2003) Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27:313–339

Park JE, Schlegel HG, Rhie HG, Lee HS (2004) Nucleotide sequence and expression of the ncr nickel and cobalt resistance in Hafnia alvei 5–5. Int Microbiol 7:27–34

Schippers A, Jozsa P-G, Sand W (1996) Sulfur chemistry in bacteria leaching of pyrite. Appl Environ Microbiol 62:3424–3431

Schlegel HG, Cosson JP, Baker JM (1991) Nickel hyperaccumulating plants provide a niche for nickel resistant bacteria. Bot Acta 104:18–25

Schmidt A, Haferburg G, Sineriz M, Schmidt A, Merten D, Büchel G, Kothe E (2005) Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Chemie Erde 65S1:131–144

Schmidt A, Schmidt A, Haferburg G, Kothe E (2007) Superoxide dismutases of heavy metal resistant streptomycetes. J Basic Microbiol 1:56–62

Schmidt A, Haferburg G, Schmidt A, Merten D, Gherghel F, Büchel G, Kothe E (2009a) Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. Chemie Erde 69S2:35–44

Schmidt A, Gube M, Schmidt A, Kothe E (2009b) *In silico* analysis of nickel containing superoxide dismutase evolution and regulation. J Basic Microbiol 49:109–118

Sineriz ML, Kothe E, Abate CM (2009) Cadmium biosorption by *Streptomyces sp*. F4 isolated from former uranium mine. J Basic Microbiol DOI:10.1002/jobm200700376

Singer PC, Stumm W (1970) Acid mine drainage: the rate determining step. Science 167:1121–1123

Zettler LAA, Messerli MA, Laatsch AD, Smith PJS, Sogin ML (2003) From genes to genomes: beyond biodiversity in Spain's Rio Tinto. Biol Bull 204:205–209

4. Diskussion

4.1 Schwermetallresistenz

Streptomyceten sind aufgrund ihrer guten Anpassungsfähigkeit als bodenbewohnende Organismen omnipotent, das von ihnen ausgehende flüchtige Geosmin macht den typischen Bodengeruch aus. Sie produzieren eine Vielfalt an Sekundärmetaboliten. So sind sie auch in lebensfeindlichen Habitaten zu finden, wie geogen oder anthropogen kontaminierten Böden. In langzeitkontaminierten Böden treten resistente Bodenbakterien vor allem in Symbiose oder Assoziation mit hyperakkumulierenden Pflanzen auf. Langzeitadaptationen führen nicht zu höherer Resistenz oder Koresistenz (Mengoni *et al.*, 2001, Idris *et al.*, 2004, 2006, Abou-Shanab *et al.*, 2007). Auf neu kontaminierten Böden dagegen überleben nur einzelne Stämme und bauen eine zum Teil hochresistente Mikroflora auf. Im Gessental des ehemaligen Uranabbaugebietes Ronneburg/ Thüringen wurden an ufernahen Austrittstellen alter Grubenwässer in den Gessenbach *Streptomyces*-Stämme aus dem Boden isoliert, von denen eine Vielzahl hochresistent gegenüber Schwermetallen reagierte, darunter auch ein Stamm mit der höchsten bekannten Nickelresistenz (Schmidt[1] *et al.*, 2005, 2009). Die Resistenz ist nicht immer an Plasmide gebunden; von den 3 Stämmen mit der höchsten Nickelresistenz besitzt ein Stamm kein Plasmid (Schmidt *et al.*, 2009). Durch die unmittelbare Nähe der Isolationsstellen kann Plasmidtransfer stattgefunden haben, späterer Plasmidverlust nach dem Transfer einzelner Gene bzw. größerer Plasmidabschnitte ins Chromosom sind ebenfalls möglich. Ob die nachgewiesenen Plasmide Resistenzgene tragen, muss noch nachgeprüft werden. Auch eine Untersuchung schwermetallresistenter Bakterien, die in Assoziation mit in kontaminiertem Wasser lebenden Schwämmen isoliert wurden, zeigte keine Korrelation zwischen der Anzahl der Plasmide und dem Grad der Resistenz (Selvin et al., 2009).

Actinobacterien und insbesondere Streptomyceten zeigen mit verschiedenen intrazellulären und extrazellulären Mechanismen eine variable Resistenz gegenüber verschiedenen Schwermetallen, weshalb sie häufig als Isolate an kontaminierten Standorten auftreten (Schmidt *et al.*, 2009, Kothe *et al.*, 2010). Eine Abwehrstrategie ist die extrazelluläre Komplexierung von Schwermetallen in der Umgebung, welche die Bioverfügbarkeit herabsetzen und damit auch assoziierten Nachbarorganismen zugute kommt. So ermöglichen chelatierende Substanzen von *S. tendae* F4 auf Cadmium-haltigen Medien das Wachstum benachbarter sensitiver Stämme (Schmidt *et al.*, 2005, 2007). In Cadmium-haltigen Medien

[1] Aufgrund des häufigen Familiennamens Schmidt, wurden eigene Erstautor-Publikationen mit Stern* markiert.

sekretiert *S. tendae* F4 drei Hydroxamat-Siderophore, die die Aufnahme von Cd in die Zellen reduzieren (Dimkpa *et al.*, 2009a, b). Verschiedene Siderophore wurden auch für *S. acidiscabies* E13 nachgewiesen, in beiden Stämmen sind sie neben der Fe-Bindung in der Lage auch verschiedene Schwermetalle wie Aluminium oder Nickel zu binden, um damit eine Vielfalt an Resistenzen zu ermöglichen (Dimkpa *et al.*, 2008a, b). Nach Untersuchungen von *S. tendae* F4 durch Siñeriz *et al.* (2009) wurden darüber hinaus eine Biosorption des Cadmiums an die Zellwand sowie die intrazelluläre Bindung an Metallothioneine diskutiert. Weitere Mechanismen, um die Bioverfügbarkeit toxischer Metalle zu verringern, sind die Reduktion oder die Oxidation. Verschiedene *Streptomyces*-Isolate aus dem durch Industrieabfälle belasteten Salí River (Tucumán, Argentinien) sind hochresistent gegenüber Chrom(IV). Durch enzymatische Reduktion zu Chrom(III) wird die Löslichkeit dieses Schwermetalls herabgesetzt, womit Anwendungen wie Bioremidiation möglich werden (Polti *et al.*, 2007). Die Resistenz gegenüber Kupfer beruht ebenfalls auf diesem Prinzip, einige Stämme besitzen eine extrazelluläre Kupferreduktase, die Cu(II) in das immobile Cu(I) überführt (Albarracín *et al.*, 2008). Die Autoren diskutieren außerdem einen Effluxmechanismus für Kupfer bei *Streptomyces sp.* AB2A, der auch für andere kupferresistente Bakterien beschrieben wurde. Funktionelle Untersuchungen zu möglichen Effluxtransportergenen in Actinobakterien stehen noch aus (Haferburg und Kothe, 2007). Andere Resistenzstrategien führen zur intrazellulären Anreicherung von Schwermetallen. Im Falle des multipel resistenten Stammes *S. acidiscabies* E13 konnten darüberhinaus auf nickelhaltigen Festmedien bis 100 µm große, grüne Kristalle als Biominerale, sogenannte Nickel-Struvite, nachgewiesen werden (Haferburg *et al.*, 2008). In der Bandbreite der sensitiven bis resistenten Organismen spielt der intrazelluläre Umgang mit durch Schwermetalle verursachte reaktive Oxidationsprodukte (ROS) eine große Rolle. Die Besetzung mit Katalasen, Peroxidasen und Superoxiddismutasen (SOD) spielt neben den direkten Resistenzfaktoren eine große Rolle (*Schmidt *et al.*, 2007).

4.2 Rolle von SOD-Isoenzymen in *S. acidiscabies* E13 in schwermetallhaltiger Umgebung

Der Stamm *S. acidiscabies* E13 wurde ebenfalls im Gessental isoliert (Amoroso *et al.*, 2000). Er wurde aufgrund seiner multiplen Resistenz gegenüber Schwermetallen ausgewählt. Sein *sodN*-Gen ähnelt den bekannten *Streptomyces sp. sodN*-Genen zu 93%, den bekannten *Streptomyces*-NiSOD-Sequenzen zu 95% (*Schmidt *et al.*, 2007). Durch Aktivitätsfärbung nach Proteingelelektrophorese konnte die Induzierung der NiSOD-Synthese nicht nur nach Wachstum mit Nickel sondern auch mit weiteren Schwermetallen nachgewiesen werden (*Schmidt *et al.*, 2007). Die Synthese der ebenfalls vorhandenen, eisenhaltigen

Superoxiddismutase wird bei *S. acidiscabies* E13 mit der NiSOD-Synthese unterdrückt. Da unter Schwermetall-Einfluss in der Zelle auch Superoxidanionen gebildet werden, kann man beide SOD-Typen als Schwermetall-Resistenz vermittelnde Proteine betrachten. Allerdings wird die NiSOD unter Stressbedingungen gegenüber der FeZnSOD eindeutig bevorzugt. Möglicherweise ist die NiSOD im Umsatz effektiver (sechs aktive Zentren statt vier) oder die Proteinstruktur der FeZnSOD ist unter Schwermetalleinfluss durch oxidative Angriffe instabiler. Solche direkten Vergleiche beider SOD sind nicht verfügbar. Andererseits werden in Eisenmangelsituationen Eisenionen auch in anderen Enzymen als Cofaktoren benötigt und durch die Einschaltung eines Nickelenzyms mit der gleichen Funktion eingespart (Dupont *et al.*, 2008a). Für die alternative Funktion beider Superoxiddismutasen muss ein direkt oder indirekt nickelabhängiger Transkriptionsfaktor vorliegen. Für *sodF* sind nickelbindende Transkriptionsregulatoren bereits bekannt. Ein direkter Einfluss auf die Transkription der *sodN* durch Nur bzw. SrnR/Q, oder andere Regulatoren wurde nicht gezeigt. Nickel tritt als zentrales Regulierungselement auch bei der Prozessierung der NiSOD-Präproteine zum Holoenzym auf.

Nicht nur die NiSOD, auch die FeZnSOD der Streptomyceten stellt scheinbar eine Einzigartigkeit dar. Kim *et al.* (1996) fanden in *S. coelicolor* Müller eine als Tetramer vorliegende SOD mit 22,2 kDa Untereinheiten, die 0,36 mol Eisen und 0,26 mol Zink pro Monomer enthält. Andere Merkmale zur Unterscheidung der FeZnSOD und der FeSOD anderer Organismen auf genetischer sowie Proteinebene sind nicht gegeben. Somit stellt sich die Frage nach Evolution, Funktion und Cofaktorbindestellen dieser zinkhaltigen FeSOD. Kirby *et al.* (1981) untersuchten die FeSOD von *Methanobacterium bryantii* und konnten 2,7 mol Eisen, 1,7 mol Zink und weniger als 0,2 mol Mangan pro Untereinheit messen. Da Zink eine hohe Affinität für apoMnSOD und apoFeSOD aufweist, vermuten die Autoren, dass Zink keine Funktionalität besitzt, sondern die Cofaktorbindestellen besetzt oder unspezifisch an das Protein bindet. Auch Youn *et al.* (1996b), die beide SOD von *S. griseus* beschrieben, stimmen diesen Schlussfolgerungen zu und nehmen an, Zink könnte die Cofaktorbindung stabilisieren, ähnlich der CuZnSOD. Für die beschriebene FeZnSOD und andere FeSOD kann ähnliches spekuliert werden. Möglicherweise besitzt die FeSOD auch durch diese unspezifische Besetzungsmöglichkeit der Cofaktorbindestelle eine geringere Aktivität als die streng Cofaktor-spezifische NiSOD.

4.3 Regulation der SOD-Genexpression

Die Synthese der FeSOD von Streptomyceten wird signifikant durch Nickel unterdrückt. Nickel greift über den SrnR/Q-Komplex bzw. den Nur-Faktor in die Regulation der

Transkription der *sodF* ein (Kim *et al.*, 1998a). Gleichzeitig stimuliert Nickel die Synthese der NiSOD, die Funktionalität der gehemmten FeSOD wird damit komplementiert (für *S. acidiscabies* E13: Schmidt *et al.*, 2005, Lischke, 2008).
Für den multipel schwermetallresistenten Stamm *S. acidiscabies* E13 konnte eine dem Nickel vergleichbare Wirkung mit weiteren Schwermetallen auf *sodN und sodF* nachgewiesen werden (Kupfer, Chrom, Mangan) (*Schmidt *et al.*, 2007). Möglicherweise wirken neben Nickel auch andere Schwermetalle indirekt *cis*-transkriptional auf *sodF*. Eventuell können sie wie Nickel an spezielle Transkriptionsregulatoren wie SrnR/Q oder Nur binden.

Das Regulatorpaar SrnR/Q (Abb. 5) konnte bisher nur für *S. griseus* nachgewiesen werden (Kim *et al.*, 2003b). Das nickelbindende Protein SrnQ weist keine Homologien zu bekannten Proteinen auf, SrnR dagegen zeigt Homologien zu anderen Transkriptionsregulatoren wie ArsR von *S. coelicolor* M145/A3(2) (52% Sequenzähnlichkeit) oder MerR von *S. lividans* (57%) (Kim 2003b). Aktuelle Datenbankanalysen zeigen Ergebnisse mit hoher Identität (95-100%) zu SrnR von *S. griseus* (114 AA) nur für *S. roseosporus* NRRL15998, *S. griseus subsp. griseus* NBRC 13350 und *Streptomyces sp.* ACT-1. Jedoch werden für eine große Zahl an Streptomyceten Proteine der ArsR-Familie mit bis zu 59% Identität ausgewiesen.
Das argininreiche SrnQ (110 AA) dagegen scheint völlig einzigartig in *S. griseus* kodiert zu sein (Kim *et al.*, 2003b). Für die N-terminalen 38 Aminosäuren gibt es wiederum in *S. griseus subsp. griseus* NBRC 13350, *Streptomyces sp.* ACT-1, *S. roseosporus* NRRL 15998) 97-100% Übereinstimmung, auch diese Sequenz scheint nur hier kodiert zu sein. Möglicherweise ist die bei *S. griseus* beschriebene Überlappung beider Gene (Kim *et al.*, 2003b) ein Hinweis darauf, dass in anderen Spezies eine stärkere Genverschiebung stattgefunden hat, wodurch die Funktion und weitere Kodierung verloren gegangen ist. SrnR zeigt in Deletionsmutanten für *srnQ* keine Repressorwirkung am *sodF*-Promotor (Kim *et al.*, 2003b).

Der Regulator Nur wirkt als Dimer und kann wie SrnQ Nickel binden (Ahn *et al.*, 2006, An *et al.*, 2008, 2009). Nach Datenbankanalysen tragen alle verfügbaren Streptomyceten, auch *S. griseus* (Identität meist 100%, einige mit 91-97%), wie auch andere Actinobakterien (87-93% Identität) dieses nickelbindende Protein der Fur-Familie. Anzunehmen ist also, dass vorwiegend Nur als Repressor am *sodF*-Promotor wirkt. Möglicherweise tritt es auch in *S. griseus* mit SrnR/Q in Konkurrenz, wenn es nicht selbst reprimiert ist.

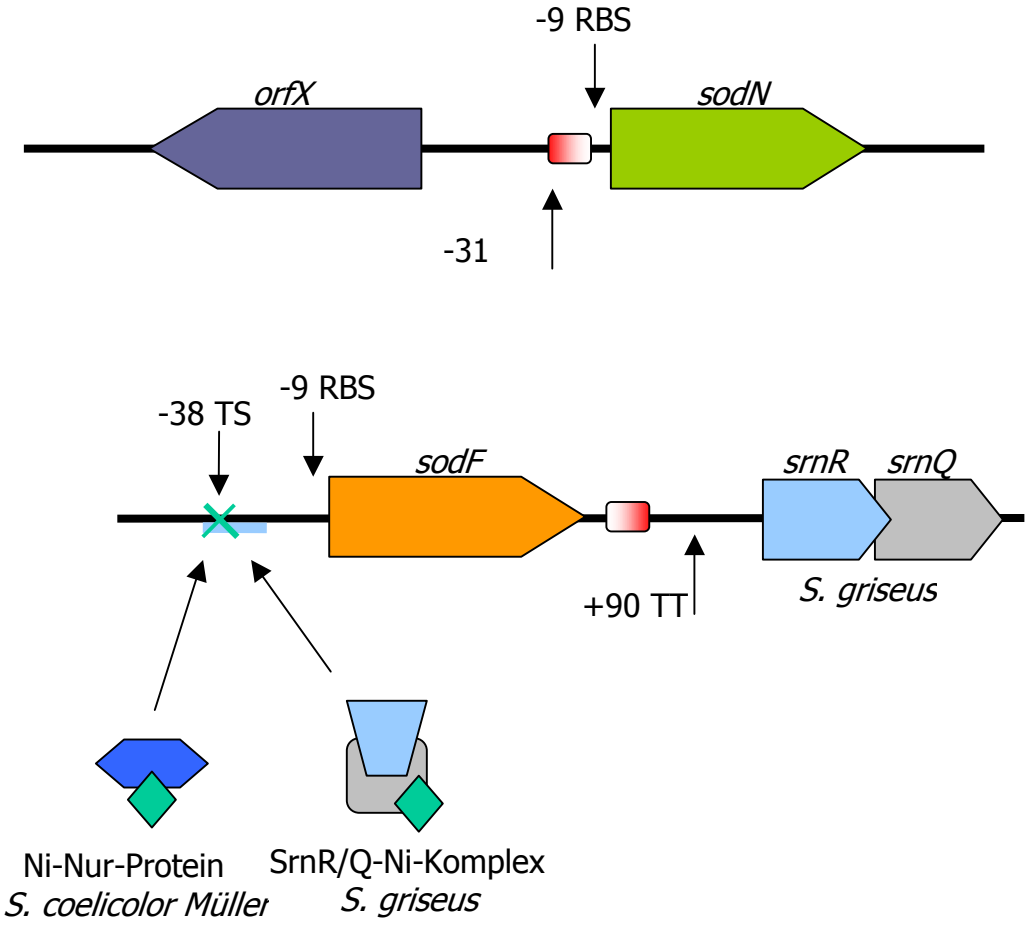

Abb. 5: Schematische Darstellung der kodierenden Regionen um *sodN* und *sodF* von Streptomyceten, der SrnR/Q-Komplex von *S. griseus* ist eingefügt, besonderes Augenmerk gilt dem 19bp-Element ▭ (TS: Transkriptionsstart, TT: Transkriptionstermination, RBS: mögliche Ribosomenbindestelle, Inverted repeat im *sodF*-Promotor ▬ , Nickel: grüner Rhombus)

4.4 Identifizierung neuer regulatorischer Elemente

In direkter Assoziation zu *sodN* und *sodF* und ausschließlich in Streptomyceten wurde eine homologe 19 Basenpaar-Sequenz identifiziert, die bei *sodN* am Transkriptionsstartpunkt (+1) und in inverser Richtung downstream der *sodF* in 14-25 Nukleotiden Abstand zum Translations-Stoppcodon liegt (*Schmidt *et al.*, 2009). Damit ergibt sich ein offensichtlicher Bezug zur Hemmung der FeSOD bei gleichzeitiger Synthese der NiSOD. Interessanterweise liegt bei *S. griseus* SrnR/Q downstream zu *sodF* und dessen 19bp-Element (160bp). Teilbereiche von bis zu 17 homologen Nukleotiden können ebenfalls benachbart zu proteinkodierenden Gensequenzen in anderen Organismen gefunden werden. *S. coelicolor* A3(2) etwa weist 48bp zur 3'-Region des 50S-ribosomalen Proteins L13 eine Homologie von 14 Nukleotiden auf. *Planctomyces limnophilus* DSM 3776 besitzt eine 16nt-Homologie 12bp zur 5'-Region des Tetrazyklin-Resistenzproteins (major facilitator superfamily MFS_1) sowie

eine 14nt-Homologie 16bp zur 3'-Region des Hitzeschockproteins DnaJ. Entsprechende Transkriptionsregulatoren für diese Proteine sind hinlänglich nicht bekannt.

Für eukaryotische Gene wurden Enhancer und Silencer beschrieben, die als *cis*-Elemente vor, hinter oder direkt im Gen liegen und die Aktivität der RNA-Polymerase am Promotor steuern. Auch für Bakterien wurden Enhancer-ähnliche Elemente beschrieben (Xu und Hoover, 2001). Die Aktivierung der RNA-Polymerase am Promotor mit dem Enhancer-Element erfolgt über Enhancer-bindende Proteine (EBP), von denen bereits eine Vielzahl für Bakterien bekannt sind. Streptomyceten besitzen eine große Vielfalt an Sigma-Faktoren, *S. coelicolor* kodiert 63 (Wigneshweraraj *et al.*, 2008), zweitplatziert ist *Mezorhizobium loti* mit 23 (Ikeda *et al.*, 2003). Die ATP-abhängige Sigma54-RNA-Polymerase ähnelt in ihrer Funktionsweise der eukaryotischen RNAPII und spielt in der Enhancer-vermittelnden Transkription eine Rolle. Das Gen für Sigma 54 ist in etwa 60% der Bakterien kodiert. Jedoch konnten Bentley *et al.* (2002) beim Screening des Gesamtgenoms von *S. coelicolor* A3(2) trotz der Vielfalt keinen Sigma-54-Faktor oder verwandte Aktivatoren finden. Er findet außerdem keine Erwähnung im Genomscreening von *S. avermitilis* (Omura *et al.*, 2001, Ikeda *et al.*, 2003) sowie *S. griseus* (Ohnishi *et al.*, 2008, auch Hsiao und Kirby, 2007). Die Proteinsuche bei NCBI nach ‚Sigma 54' in *Streptomyces* ergibt jedoch zahlreiche Einträge für ‚Sigma-54 modulation proteins' und ‚Sigma-54 interacting domain proteins'. Möglicherweise sind dazugehörige Sigma 54-Faktoren bei Streptomyceten verloren gegangen. Für *S. coelicolor* A3(2) liegt an Position SCO_3623 ein Transkriptionsregulator, der Ähnlichkeit zu *luxZ* aufweist, dem Enhancer des *lux*-Operons von *Photobacterium leiognathi*. Die endgültige Klärung, ob es Enhancer-vermittelte Transkriptionsregulation bei Streptomyceten gibt, steht demnach noch aus.

Eine mögliche Erklärung der lokalen Nähe des 19-Basenpaar-Elements zu den *sod*-Genen und dessen doppeltes Vorkommen im Genom bietet die mRNA-Hybridisierung (Abb. 6). Bei geringen Mengen von Nickel sind nach Anfärbung aktiver Proteingele beide SOD-Typen zu beobachten. Bereits geringste Mengen Nickel induzieren die *sodN*-Transkription. Gleichzeitig mit dem Gen für *sodF* wird also auch bereits das *sodN*-Gen transkribiert. Stufenweise Erhöhung der Nickelmenge im Medium erhöht schrittweise die Menge aktiver NiSOD und reduziert gleichzeitig die FeSOD-Menge (Kim *et al.*, 1996). Das 19bp-Element liegt auf beiden Transkripten, ist demnach auf der mRNA kodiert. Würde es zur Hybridisierung des *sodN*-19bp-Elements mit dem invers kodierten *sodF*-19bp-Element kommen, wäre die Ribosomenbindestelle der *sodN* blockiert und damit die Translation unterdrückt. Sobald größere Mengen Nickel in die Zelle gelangen, wird die Transkription des *sodF*-Gens durch

Nur bzw. SrnR/Q vollständig gehemmt. Die *sodN*-mRNA wird dagegen synthetisiert und kann zur NiSOD translatiert werden, wobei weitere nickelsensitive Aktivatoren eine Rolle spielen könnten. Das 19bp-Element könnte somit eine Rolle in der Feinjustierung der *sodN*-Translation spielen, um erst ab einer bestimmten Nickelkonzentration die aufwändige NiSOD-Synthese zu starten.

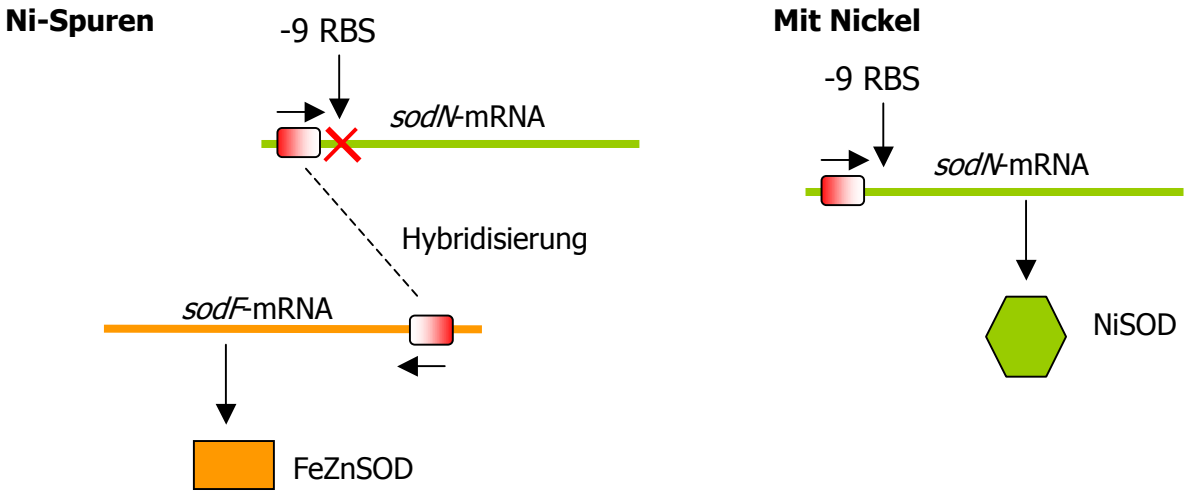

Abb. 6: Mögliche Erklärung der gegensätzlichen nickelabhängigen Regulation der FeZnSOD und NiSOD bei Streptomyceten durch mRNA-Hybridisierung (RBS: mögliche Ribosomenbindestelle)

Promotorbindende Proteine werden mittels EMSA (electromobility shift assay) gesucht, nach dieser Methode wurde unter anderem Nur als *sodF*-Repressor gefunden (Ahn *et al.*, 2006). Erste Durchführungen dieser Methode mit dem 19bp-Element und *S. lividans* TK23 ergaben mittels Fluoreszenz-EMSA mögliche promotorbindende Aktivitäten (Koch, 2010).

4.5 Vorkommen verschiedener SOD kodierender Gene

Für die nickelbedingte Regulation der *sodF* wurden zwei verschiedene Mechanismen gefunden. In *S. griseus* hemmt ein Komplex aus SrnR und SrnQ die FeSOD-Transkription (Kim *et al.*, 2003b). In *S. coelicolor* Müller dagegen ist ein Nur-Protein der *sodF*-Repressor (Ahn *et al.*, 2006). Potentielle ArsR bzw. SrnR-ähnliche Proteine werden im Genom von *S. coelicolor* A3(2) kodiert (Kim *et al.* 2003b), der außerdem zwei unabhängige FeSOD besitzt (Chung *et al.*, 1999a). FeSOD1 ähnelt zu 100% der FeSOD von *S. coelicolor* Müller, beide werden schon bei Spuren von Nickel gehemmt (0,5 µM bzw. 1µM $NiCl_2$, Chung *et al.*, 1999a, b). FeSOD2 zeigt nur 88% Sequenzähnlichkeit und ist erst bei 10 µM bzw. 100 µM $NiCl_2$ vollständig unterdrückt. NiSOD-Aktivität kann jeweils ab etwa 1 µM $NiCl_2$ beobachtet werden. Augenscheinlich wurden hier nach Genduplikation von *sodF* verschiedene Regulationsmechanismen etabliert.

Streptomyceten besitzen mehrheitlich jeweils eine NiSOD und eine FeZnSOD. Während *S. coelicolor* A3(2) zwei FeSOD kodiert, scheinen einige Streptomyceten nur die NiSOD zu enthalten, wie *S. albus* KCTC9006 und *S. longisporoflavus* (Leclere *et al.*, 1999, Youn *et al.*, 1996). Die Autoren nutzten YEME-Medium ohne Zusatz von Nickelsalzen. Allerdings ist nicht auszuschließen, dass die FeSOD-Synthese in beiden Stämmen schon bei geringsten Nickelspuren gehemmt wird (Spurenmetalle in Medien, Kim *et al.*, 1996). *S. longisporoflavus* ist in keinem Genomprojekt beteiligt, für den Stamm *S. albus* J1074 werden allerdings in Datenbanken sogar drei SOD-Typen angegeben: eine potentielle NiSOD (131 AA, ZP_06593476.1), eine potentielle FeSOD (212 AA, ZP_06590720) und zwei potentielle CuZnSOD (196 AA, ZP_06590491.1 und 213 AA, ZP_04701747.1) mit jeweils 51% Identität zur CuZnSOD von *Frankia sp.* CcI3 (194 AA). In der Literatur wird für Streptomyceten keine CuZnSOD erwähnt, durch Datenbankanalysen kann man jedoch mögliche CuZnSOD-Proteine finden. So besitzen *Streptomyces sp*. AA4, *Streptomyces sp.* ACT-1, *Streptomyces sp.* SPB74, *S. bingchenggensis* BCW-1, *S. griseus subsp. griseus NBRC 13350*, *S. hygroscopicus* ATCC 53653 und *S. roseosporus* NRRL 15998 potentielle CuZnSOD mit 48-56% Identität zur CuZnSOD von *Frankia sp.* CcI3 und 41-98% innerhalb der Streptomyceten-CuZnSOD. Die Länge der Proteine differiert zwischen 195-263 Aminosäuren. Weitere ähnliche CuZnSOD werden ausschließlich in verschiedenen Actinobakterien gefunden.

CuZnSOD wird in eukaryotischem Cytosol, extrazellulär in Mammalia sowie in Chloroplasten gefunden (Imlay und Imlay, 1996). Das Enzym wurde inzwischen auch für viele Bakterien beschrieben. Für *E. coli* jedoch waren nach fast 20 Jahren intensiver SOD-Forschung nur der Besitz aktiver MnSOD und FeSOD bekannt. Erst Benov und Fridovich (1994) fanden in einer *sodA*-*sodB*-Nullmutante eine dritte SOD-Aktivität, die CuZnSOD, kodiert durch *sodC*. Dieses Enzym ist im Periplasma lokalisiert, Wärme- und pH-sensitiv (Instabilität in Zellextrakten) und zudem nicht häufig (in der log-Phase fast komplett reprimiert) (Benov und Fridovich, 1994). Diese Eigenschaften könnten die bisherige Nichterwähnung einer CuZnSOD für Streptomyceten erklären. Da diese Gruppe im Gegensatz zu *E. coli* grampositiv ist, ist auch die Untersuchung der potentiellen *sodC* auf die Leadersequenz und damit die Lokalität und Aktivität in verschiedenen Wachstumsphasen neben den anderen SOD-Typen von Interesse.

Die fehlende FeSOD (Leclere *et al.*, 1999, Youn *et al.*, 1996b) könnte somit bei Nickelmangel durch eine CuZnSOD-Aktivität kompensiert werden. Bei einigen *Streptomyces spp.* kann die fehlende FeSOD-Aktivität durch Sequenzverluste im *sodF*-Gen erklärt werden. So besitzt *S. griseoflavus* Tu4000 nur die zu 70% homologe C-terminale Teilsequenz (ZP_05540676.1) der *S. coelicolor* A3(2) *sodF* (SCO_2633), mit nur 86/213 Aminosäuren. *S. bingchenggensis* kodiert zwei FeSOD (ADI05625.1 und ADI04378.1), letztere zeigt aber einen zentralen Sequenzverlust von 110 Aminosäuren. Dupont *et al.* (2008a) fanden in zwei Cyanobakterien

der Gattung *Synechococcus* eine auffallende Syntänie in den Genregionen um *sodB* (FeSOD, Stamm WH7803) bzw. *sodN* (Stamm WH8102), die auf einen direkten Austausch von *sodB* gegen *sodN*|*sodX*|*sodT* hinweist (Abb. 7). Das zeigt einerseits den funktionellen Zusammenhang der drei Gene *sodN*|*sodX*|*sodT*, andererseits besitzt *Synechococcus* WH8102 nur diese NiSOD, Stamm WH7803 diese FeSOD und eine CuZnSOD (Priya *et al.*, 2007). Dupont *et al.* (2008a) diskutieren den starken Druck, Eisenmangelsituationen mithilfe anderer Cofaktoren bzw. Enzyme zu auszugleichen. Es lässt sich also vorhersagen, dass Organismen, die nur noch die NiSOD besitzen, unter aeroben Bedingungen stark nickelabhängig sein sollten.

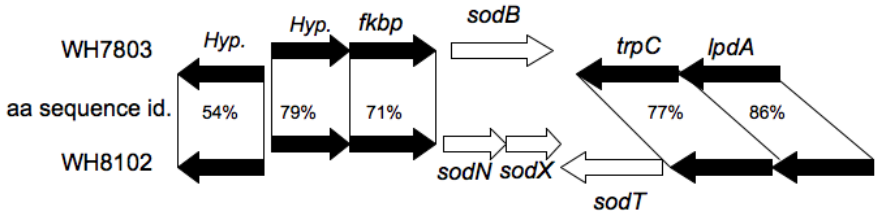

Abb. 7: Darstellung der Syntänie zweier Genregionen von *Synechococcus* WH8102 und WH7803, *sodB*: FeSOD, *sodN*: NiSOD, *sodX*: Peptidase, *sodT*: Nickeltransporter, *fkbp*: Isomerase (Dupont *et al.*, 2008a, Fig. S3)

4.6 Verbreitung und Evolution *sodN*

Die Differenzen zu anderen Superoxiddismutasen führten zu der Frage nach der Herkunft und Evolution der NiSOD. NiSOD wurden zunächst nur für Streptomyceten beschrieben (Youn *et al.*, 1996a, b, Kim *et al.*, 1996) und in diesen Organismen auch genetisch sowie strukturell untersucht (Barondeau *et al.*, 2004, Wuerges *et al.*, 2004). Durch Gesamtgenom-Sequenzierungen wurden auch in marinen Cyanobakterien *sodN*-Gene gefunden (Palenik *et al.*, 2003, 2006). Durch *in silico*-Analysen (Dupont *et al.*, 2008a, *Schmidt *et al.*, 2009) konnte die Verbreitung der NiSOD-Gene in weiteren Bakteriengruppen (Actinobakterien, Proteobakterien, *Chlamydiae*) und *Eukarya* (Grünalgen) nachgewiesen werden. Aktive NiSOD-Proteine wurden bislang nur in verschiedenen *Streptomyces*-Stämmen, zwei *Synechococcus*-Stämmen (Dupont *et al.*, 2008b) sowie für *Prochlorococcus marinus* MIT9313 (Eitinger, 2004) gezeigt, wobei die Aktivität der *Prochlorococcus*-NiSOD nur in *E. coli* analysiert wurde. Cyanobakterien haben statt 14 eine 19-26 Aminosäuren lange N-terminale Sequenz, die fertigen Untereinheiten bestehen je aus 132-137 Aminosäuren.

Das Auftreten des *sodN*-Gens in allen Actinobakterien, aber nicht in nah verwandten Bakterien, weist auf einen heterologen Gentransfer hin. Da ein Großteil der Organismen mit möglichen *sodN*-Genen in marinem Bereich lebt, scheint hier der evolutionäre Ursprung zu liegen. Andererseits könnten bodenbewohnende *sodN*-tragende Organismen durch

geologische Abrutschungen ins Meer und bis in die Tiefsee gelangt sein, wo schließlich Gene horizontal weitergegeben wurden. Metagenomanalysen stellten heraus, dass sämtliche angenommenen *sodF*/*sodN*-Austauschereignisse ausschließlich in marinen Isolaten gefunden haben, *sodN* taucht in nicht-marinen Metagenomen bis auf die Gruppe der Actinobakterien nicht auf (Dupont *et al.*, 2008a). Diese Erkenntnisse führen die Autoren zu der Hypothese, dass die NiSOD vor allem durch umweltbedingten Stress, besonders durch geringe Eisenverfügbarkeit, bevorzugt wird.

Priya *et al.* (2007) analysierten die 64 in Datenbanken zugänglichen Cyanobakterien und kamen zu dem Ergebnis, dass primitive einzellige *Prochlorococcus spp.* mit einfachem Photosyntheseapparat nur den nickelhaltigen SOD-Typ kodieren, etwas komplexere Photosynthese betreibende Cyanobakterien enthalten eine Kombination aus MnSOD-/ NiSOD-Genen oder FeSOD-/ MnSOD-Genen, während die höchstentwickelten Formen nur Gene für FeSOD und MnSOD tragen, CuZnSOD-Gene kommen nur sehr selten vor. Möglicherweise ist dies ein Hinweis auf horizontalen Gentransfer von *sodN* mariner Streptomyceten zu einfachen Cyanobakterien, wie auch primitiven Eukarya. Organismen mit kleiner Genomgröße könnten danach zur effektiven Ressourcennutzung Gene anderer SOD verloren bzw. rearrangiert haben, wie bei *Ostreococcus tauri* für die NiSOD beschrieben (*Schmidt *et al.*, 2009). Die Grünalge *O. lucimarinus* besitzt neben der NiSOD auch ein MnSOD-Gen (Gen-ID: 5001449, *sodA*) und ein Gen für die CuZnSOD (NC_009373, OSTLU_28559, predicted protein). Der Cyanobakterien-Stamm *Synechococcus sp.* CC9311 besitzt zwei mögliche NiSOD, allerdings mit nur 14,8% Aminosäuresequenz-Identität. Während SodN1 (YP_729969, Sync_0755) Homologien zu anderen cyanobakteriellen NiSOD zeigt, besitzt SodN2 (YP_731630 Sync_2434) 56,4% Identität zur NiSOD des Proteobakteriums *Desulfotalea psychrophila* NiSOD (*Schmidt *et al.*, 2009). Dieses Bakterium lebt in arktischen marinen Sedimenten (Svalbard/ Spitzbergen, Knoblauch *et al.*, 1999, Rabus *et al.*, 2004) gegenüber *Synechococcus sp.* CC9311, der aus kalifornischen Küstengewässern isoliert wurde (Toledo und Palenik, 1997, Palenik *et al.*, 2006). Die Evolution bezüglich Zeitpunkt und Lokalität dieser NiSOD, der so verschieden umweltadaptierten Organismen bleibt aufzuklären.

Im Boden ist Nickel in Mengen vorhanden, die den Vorkommen von Kupfer und Zink vergleichbar sind (Kim *et al.*, 1996). Demgegenüber ist Nickel im oberflächennahen Meereswasser nur im nanomolaren Bereich vorhanden, die Konzentration steigt mit zunehmender Tiefe (‚nährstoffähnliches' Tiefenprofil, Dupont, 2008b). Viele marine Organismen des Phytoplanktons besitzen eine Ni-haltige Urease (Stickstoffassimilation),

einige Cyanobakterien tragen außerdem eine Ni/Fe-Hydrogenase. Durch den Besitz mehrerer Nickelenzyme sind sie nickelabhängig im Wachstum. Dupont *et al.* (2008b) beobachteten, dass *Synechococchus sp.* in Medien mit Harnstoff ohne Nickel im Wachstum gehemmt ist und keine SOD-Aktivität gemessen werden kann. Nach dem Wechsel der N-Quelle zu Ammonium (NH_4^+) ist die SOD-Aktivität leicht erhöht, was durch eine Relokalisierung von Nickel aus der Urease zur NiSOD erklärt wird. Die NiSOD und FeSOD der Streptomyceten sind vermutlich cytoplasmatisch aktiv, werden aber antagonistisch reguliert. Sobald Nickel vorhanden ist, wird die NiSOD angeschaltet. Bekannte marine Cyanobakterien jedoch besitzen nie NiSOD- und FeSOD-Gene gemeinsam (Priya *et al.*, 2007). Der Ersatz der FeSOD durch die NiSOD führt zu einer geringeren Abhängigkeit von Eisen, dass im Meereswasser stark limitiert vorkommt.

Die meisten Streptomyceten scheinen ebenfalls mehrere Proteine mit Nickelbindestellen zu besitzen: NiSOD, Urease und NiFe-Hydrogenase (Ikeda *et al.*, 2003, Bentley *et al.*, 2002), außerdem Nur bzw. SrnQ. Die intrazelluläre Nickel-Konkurrenz bei gleichzeitiger Schadensabwehr durch das Schwermetall muss demnach reguliert werden und hat Ausschlag auf das Wachstum der Zellen. Nickelbindende Metallochaperone wie ureE könnten der NiSOD Nickelionen wegfangen (Dupont 2008b). Das Wachstum auf stark harnstoffhaltigen Böden (Gülle) könnte demnach für Bodenorganismen, wie Streptomyceten, ebenfalls limitiert sein.

4.7 Struktur der NiSOD

Die Funktionalität der NiSOD wurde in den meisten *sodN*-kodierenden Organismen noch nicht nachgewiesen. Vergleiche der Nickel-Bindestellen in der Protein-3D-Struktur zeigten jedoch hohe Homologien im aktiven Zentrum und in strukturgebenden bzw. funktionsgebundenen Sequenzen. *Schmidt *et al.* (2009) verglichen 49 potentielle NiSOD-Sequenzen aus verschiedenen Organismengruppen, neben Bakterien verschiedener Gruppen auch Eukaryoten. Alle Präproteine zeigen hohe Homologie im N-Terminus, der möglichen Prozessierungsstelle (splicing site) und im Nickelhaken. Allein die NiSOD der beiden Algen zeigen ungewöhnlich lange N-Termini. Aus Strukturuntersuchungen der NiSOD bekannte Sequenzen, die für Helices oder andere strukturgebende Regionen erforderlich sind, zeigten hohe Ähnlichkeiten, was auf die Funktionalität der potentiellen Enzyme schließen lässt, sowie deren Synthese, soweit die diskutierten NiSOD-assoziierten Proteine ebenfalls synthetisiert werden.

Vergleicht man die Struktur potentieller NiSOD fällt die Übereinstimmung der Nickel koordinierenden Liganden HCXXC auf (*Schmidt *et al.*, 2009, Dupont *et al.*, 2008a). Gegenüber der hohen Konservierung des Nickelhakens variieren die Aminosäurereste der Außenhülle (outer sphere) stark. Die Ligandenumgebung scheint vor allem die Funktion zu

erfüllen, das für die Dismutation nötige Redoxpotential von ~290mV bereitzustellen (Herbst *et al.*, 2009). Darin zeigt die NiSOD Übereinstimmung mit den anderen SOD-Isoformen.

Gegenüber aktuell wenig Literatur zur Regulation der NiSOD wurde das Enzym und vor allem der Nickelhaken mit dem aktiven Ni-Zentrum eingehend strukturchemisch untersucht um den ungewöhnlichen Mechanismus besser zu verstehen. Von Interesse ist einerseits die einzigartige Einbeziehung von Thiolaten (S-Liganden von Cys2 und Cys6) in die Koordinierung des Ni-Cofaktors und deren Schutz vor Oxidation während des katalytischen Zyklus. Außerdem wird die Art des Elektronentransfers (inner bzw. outer sphere) des Superoxids am aktiven Zentrum untersucht. Weiterhin ist noch unklar, ob das aktive Zentrum im reduzierten Zustand tatsächlich planar koordiniert ist oder der axiale Ligand (His1) bestehen bleibt.

Zur Funktionsaufklärung der beteiligten Liganden wurden einzelne Aminosäuren des Nickelhakens mutiert, um die Nickelbindung, Hakenbildung und katalytische Aktivität zu vergleichen. Ryan *et al.* (2010) mutierten Cys2 und Cys6 zu Ser. Die Mutanten bildeten ein Hexamer mit Nickelbindung in 1:1-Stöchiometrie wie im Wildtyp, allerdings ohne Aktivität. Jede Einzel-Cys-Mutation verhindert die natürliche Koordination des anderen Cys, was zu veränderten Spin-Konfigurationen und verhinderter katalytischer Aktivität führt. Schwefel-enthaltende Moleküle sind sehr anfällig für Modifizierung und Angriff durch Oxidantien, eine schwefelbasierte Oxidation schien darum unklar (Fiedler *et al.*, 2005). Bei Untersuchungen von Ni(II)N_2S_2-Komplexe konnte gezeigt werden, dass die Reaktivität der Thiolate durch Wasserstoffbindungen zum Nickelion verschoben wird (Gale *et al.*, 2010). Daran beteiligt sind ebenfalls die Amin- und Amid-Liganden von Cys2 und Cys6; durch Kombination dieser N-Liganden werden kritische Zustände, die zum nukleophilen Angriff der S-Liganden durch Sauerstoff führen könnten, verhindert (Shearer *et al.*, 2008). Das axiale Imidazol spielt hier ebenfalls eine Rolle (Mullins *et al.*, 2009).
Neupane *et al.* (2007) untersuchten His1-Mutanten mit der Schlussfolgerung, dass der axiale Ligand entgegen früherer Vermutungen in NiSODox erhalten bleibt und gleichzeitig die Disproportionierungsreaktion des Superoxidanions erhöht. Neueren Kristallographiestudien zufolge bleibt der axiale Ligand sehr schwach bestehen und erleichtert möglicherweise die Freilassung des O_2 (Silaghi-Dumitrescu, 2009, Shearer *et al.*, 2009).
Weitere Diskussionen werden um die Lokalisierung des Elektronentransfers für die Disproportionierung geführt. Tietze *et al.* (2009) konnten ein Nickelhaken-Cyanid-Addukt isolieren, in dem Cyanid nicht nur elektrostatisch an das Peptid gebunden ist, sondern über mindestens eine Wasserstoffbindung. Sie diskutieren für die NiSOD einen inner-sphere-

Elektronentransfer zum Superoxidanion (auch Schmidt *et al.*, 2008). Diese Art des Elektronentransfers ist aufgrund der oftmals sterischen Proteinumgebung in der Biochemie eher selten. Andere Autoren postulieren dagegen einen outer-sphere-Mechanismus (Neupane *et al.*, 2007, Silaghi-Dumitrescu, 2009), sie gehen nicht davon aus, dass eine Ni-O-Bindung für den Elektronentransfer ausgebildet wird. Sie argumentieren außerdem damit, dass auch das Anion Azid nicht an das aktive Zentrum bindet, was konsistent mit den positiv geladenen Resten geht, die das Superoxid führen (Fiedler *et al.*, 2005).

Die weniger konservierte Ligandenumgebung des aktiven Zentrums hat vor allem die Funktionen, das Redoxpotential zur Ausführung der Disproportionierung von Superoxid zu stabilisieren, Protonen bereit zu stellen und die Anionen sicher zu leiten (Herbst *et al.*, 2009). Mutationsanalysen konnten auch hier die Beteiligung einzelner Aminosäuren an der Nickelkoordination, als Protonendonoren bzw. in der Strukturgebung aufklären (Szilagi *et al.*, 2004, Bryngelson *et al.*, 2004, Würges *et al.*, 2004, Schmidt *et al.*, 2008). Sequenzvergleiche nach Datenbankanalysen (Dupont *et al.*, 2008a, *Schmidt *et al.*, 2009) konnten weitere konservierte bzw. nicht übereinstimmende Aminosäurereste aufzeigen. Alle NiSOD-Präproteine zeigen die proteolytische Schnittstelle, die entsprechenden Peptidasen wurden in einigen Gruppen bereits postuliert (Eitinger, 2004, *Schmidt *et al.*, 2009). His1, Cys2 und Cys6 sind wie erwähnt über alle Organismenreiche konserviert. Asp3, Pro5 bzw. Tyr9 des Nickelhakens sind ebenfalls weitreichend konserviert, in einigen Gruppen jedoch gegen andere Aminosäuren ausgetauscht. Daraus resultierende Änderungen in Aktivität und Struktur können in zukünftigen Arbeiten durch spezifische Mutationsanalysen immitiert werden. Einige Aminosäuren, für die bereits spezielle Funktionen postuliert und berechnet wurden, wie Val8 und Glu17, die mit His1 interagieren sollen (Lee *et al.*, 2009), sind für Actinobakterien konserviert, in allen anderen Gruppen aber ausgetauscht. *In silico* Analysen leisten somit einen Beitrag in der Bestätigung postulierter und in der Erkennung weiterer konservierter Regionen der NiSOD aller Organismenreiche und können durch spezifische Strukturaufklärungen der einzelnen Aminosäureaustausche weiterführen. Von besonderem Interesse ist die Aufklärung der Prozessierung und Aktivität in allen Gruppen neben den Streptomyceten und Cyanobakterien, vor allem der Algen-NiSOD als eukaryotische Isoform.

4.8 NiSOD-assoziierte Proteine

4.8.1 Peptidase SodX

Neben den bereits beschriebenen Repressoren in der FeSOD-Regulation Nur und SrnR/SrnQ haben weitere Proteine Einfluss auf die Synthese der NiSOD. Direkt benachbart zu *sodN*

finden sich im Genom der meisten Streptomyceten sowie bei Cyanobakterien *sodX*/ *orfX* genannte Gene, die für Peptidasen aus der S24/S26-Proteinfamilie kodieren, welche Enzyme N-terminal prozessieren (*Schmidt *et al.*, 2009, Dupont *et al.*, 2008a). Auch viele Actinobakterien besitzen dieses Gen mit hoher Sequenzidentität. Eitinger (2004) wies die Aktivierung der *Prochlorococcus*-NiSOD in *E. coli* nach Kotransformierung von *sodX*/*sodN* nach, der *E. coli*-Wildtyp kann die NiSOD nicht synthetisieren. Struktur- und Sequenzähnlichkeiten zwischen cyanobakterieller und Streptomyceten-NiSOD lassen eine ähnliche Funktion des homologen Nachbargens in der Prozessierung des N-terminalen Überhangs vor His1 vermuten.

4.8.2 Trans-to-cis-Isomerase

Eitinger (2004) fand in *P. marinus* upstream zu *sodN* eine trans-to-cis-Isomerase vom FKBP-Typ und diskutiert die mögliche Beteiligung dieses Enzyms an der Nickelhakenbildung an Pro5. Vor der Prozessierung an His1 liegt der Nickelhaken geöffnet vor (trans). Nach der nickelaktivierten proteolytischen Spaltung wird der Nickelhaken durch eine trans-to-cis-Isomerisierung an Pro5 und die Ligandierung von Cys6 ausgebildet (Fig. 4, Barondeau *et al.*, 2004). In vielen Streptomyceten können Gene mit Homologie zum C-Terminus gefunden werden, der die FKBP-Superfamilie-Domäne trägt, u.a. für *S. coelicolor* A3(2) (SCO_1638) und *S. avermitilis* (SAV_6687). Die speziesübergreifende Konservierung dieser Enzyme lässt einen Einfluss in die Prozessierung der prä-NiSOD vermuten. Pro5 ist in allen NiSOD stark konserviert und nur in den beiden *Mycobacterium*-NiSOD gegen Phe5 bzw. Tyr5 ausgetauscht (*Schmidt *et al.*, 2009).

4.8.3 Nickelbindeproteine

Streptomyceten sind heterogen resistent gegen verschiedene Schwermetalle. Da einige, wie Nickel aber andererseits essentiell sind, müssen die Zellen Nickelbindeproteine zur Verfügung haben, um die Schwermetallionen zu den Zielproteinen zu geleiten. Als mögliche Nickelchelatase fanden Kim *et al.* (2003a) in *S. seoulensis* das nickelbindende Protein CbiXhp. Homologe metallbindende Proteine werden in mehreren anderen Streptomyceten gefunden, unter anderem SAV_6405 (*S. avermitilis*) oder SCO_1858 (*S. coelicolor* A3(2), mit Ähnlichkeit zur Cobaltchelatase CbiX von *Bacillus megaterium* (39%). Andere metallbindende Liganden sind Siderophore, die hauptsächlich ins Milieu ausgeschieden werden, um Eisen zu binden. Dimkpa et al. (2008b) beschrieben drei Siderophoren aus *S. acidiscabies* E13, die neben der Fe-Bindung in der Lage sind auch Nickel zu binden. Für *S. tendae* F4 konnten Siderophore gezeigt werden, die in Pflanzen-Assoziationen die pflanzliche Aufnahme von Eisen und Cadmium aus verunreinigten Böden erhöhten (Dimkpa *et al.*, 2009b). Das

Zusammenspiel der extra- und intrazellulären Nickelbindeproteine im Mechanismus der NiSOD-Synthese muss noch weiter untersucht werden.

Auch Metallothioneine und Metallohistine kommen als Nickelbindeproteine in Betracht. Metallothioneine sind relativ kleine Proteine mit hohem Cys- bzw. His-Gehalt. Während eukaryotische Metallothioneine Metalle nur über Schwefel koordinieren, nutzen Prokaryoten auch Stickstoffliganden (Blindauer *et al.*, 2007). Das zinkbindende Metallothionein SmtA des Cyanobakteriums *Synechococcus elongatus* PCC 7942 ist derzeit das bestuntersuchte bakterielle Metallothionein. Die Expression von SmtA wird durch den Transkriptionsfaktor SmtB unterdrückt (Morita *et al.*, 2003), der interessanterweise Homologien zu den ArsR-Transkriptionsregulatoren der Streptomyceten aufweist (z.B. *S. coelicolor* A3(2), SCO6459), aber nicht zu SrnR von *S. griseus*. Für Actinobakterien ist nur das Cys-reiche kupferbindende Metallothionein MymT aus *Mycobacterium tuberculosis* bekannt (Gold *et al.*, 2008). Schmidt *et al.* (2010) untersuchten Genome von Aktinobakterien *in silico* auf kurze (<100AA) Cys- und His-reiche (>15%) Peptidsequenzen und konnten 103 mögliche Metallothioneine und Metallohistine finden. Für Streptomyceten konnten Peptide gezeigt werden, die Homologien zu metallbindenden Regionen von Schwermetalltransportern, zu Zinkfingerdomänen oder zu eukaryotischen Metallothioneinen aufweisen (Schmidt *et al.*, 2010) und damit als Metallothioneine und Metallohistine funktionieren könnten. Neben diesen Peptiden gibt es weitere Proteine, die durch His-reiche Motive auffallen und als Nickelbindeproteine in Frage kommen. NikR, der Repressor des nik-Operons von *E. coli* besitzt einen His-reichen C-Terminus der bedeutend für die Ni-Bindung ist (Eitinger und Berthelot, 2000, Dosanjh und Michel, 2006). His-reiche N-terminale Motive zeigen zwei hypothetische Proteine von *S. coelicolor* A3(2) (SCO_6145 und SCO_6549), deren Funktionalität noch aufgeklärt werden muss.

4.8.4 Hochaffine Nickeltransporter

Nickel wird in bakterielle Zellen über ABC-Transporter (NikABCDE) oder Nickel-Cobalt-Permeasen (NiCoT-Familie) importiert (Dosanjh und Michel, 2006, Rodionov *et al.* 2006). Nickel kann außerdem unspezifisch durch Mg-Transporter in die Zelle gelangen (*E. coli*, Kim *et al.*, 1998a). Die NiSOD-Synthese reagiert sehr sensibel auf geringste Nickelspuren in der Umgebung, was auf hochaffine Nickeltransporter schließen lässt. Amoroso *et al.* (2000) fanden in *Streptomyces*-Stämmen (unter anderem *S. tendae* F4) *nikA*-Fragmente mit Homologie zu bekannten bakteriellen Nickeltransportern. Ahn *et al.* (2006) konnten durch Datenbank-Analysen in *S. coelicolor* A3(2) M145 ein mögliches Nickeltransporter-Gencluster SCO_6451–6455 (*nikABCDE*-Operon, auch Eitinger und Berthelot, 2000) finden, dass zudem durch Nur in Anwesenheit von Nickel durch Bindung an den *nikA*-Promotor negativ reguliert

wird. In Datenbankanalysen clustern die von Amoroso *et al.* (2000) gefundenen Fragmente jeweils im mittleren Teil der *nicT1* (SAV_560) bzw. *nicT2*-Sequenzen (SAV_2333) von *S. avermitilis*. Diese kodieren für zwei hochaffine Nickeltransportergene mit 58,2% Identität, beide liegen jedoch zwischen Genen anderer Leserichtung bzw. ohne Homologie zu bekannten Proteinen, sie weisen auch keine Homologie zu *nikA* aus *S. coelicolor* A3(2) auf. Homologe Gene zu *nicT1* und *nicT2* können nur in *S. sviceus* ATCC29083 (ZP_06921777.1 und ZP_06918496.1), *S. hygroscopicus* ATC53653 (ZP_05520010.1) und *S. scabiei* 87.22 (SCAB_84421) gefunden werden. Das als potentielles Substratbindeprotein bzw. ABC-Transporterprotein deklarierte Gen *nikA* aus *S. coelicolor* A3(2) dagegen findet sich mit 70-99% Identität in vielen anderen Streptomyceten wieder, jedoch nicht parallel zu *nikT1*/*nikT2*. Unter anderem besitzt *S. griseus subsp. griseus* NBRC 13350 ein *nikA*-Homolog (SGR_498), welches ebenfalls als erstes von fünf Transporterproteinen operonähnlich angeordnet ist.

4.8.5 Artspezifische Proteinausstattung

Wie auch schon bei den beschriebenen Regulationsproteinen (Nur, SrnR/Q) zeigt sich auch hier eine hohe Diversität innerhalb der Gattung *Streptomyces*. Streptomyceten sind unter anderem für ihre Vielfalt an Sekundärmetaboliten und ihre morphologische Diversität bekannt. Sie besitzen große Chromosomen (7845 proteinkodierene Gene in *S. coelicolor* A3(2)), die oft linear sind und für Bakterien einzigartige terminale inverted repeats (Replicons) mit kovalent gebundenen Proteinen aufweisen (Hsiao und Kirby, 2007). Die Gattung bildet ein Monophylum mit jedoch signifikanter Gendiversität. Vergleiche verschiedener Genome zeigen relativ gut konservierte Kernregionen (für *S. coelicolor* A3(2) SCO_2050-SCO_5800) gegenüber variablen terminalen Regionen (<SCO_1100 und >SCO_7600). Die Linker dazwischen scheinen artspezifische Gene und Gencluster zu kodieren (Hsiao und Kirby, 2007). Betrachtet man die Lokalität der oben beschriebenen Nickeltransportergene, Nickelchelatoren und SOD-Regulatorgene, liegen diese zum Teil außerhalb der Kernregion auf den artspezifischen Linkern und in den terminalen Regionen (Tab. 1). Sie sind somit durch Genverluste, horizontalen Gentransfer und andere Umstrukturierungen innerhalb der Gattung *Streptomyces* verschieden konserviert, was die Unterschiede in der Regulierung identischer Gene der Kernregion erklären kann. Chen *et al.* (2002) diskutieren außerdem die unsymmetrische Linearisierung des ursprünglich zirkularen Chromosoms. Daraus resultiert eine Verlagerung der stärker konservierten Genregionen aus dem Chromosomenzentrum in Richtung der größeren Änderungen unterworfenen Chromosomenenden, womit wiederum verschiedene Genverluste unter den *Streptomyces*-Arten erklärt sein könnten.

Tab. 1: Übersicht der diskutierten Proteine bezüglich ihrer Lage auf den Chromosomen, Regionen nach Hsiao *et al.*, 2007 (SCO: *S. coelicolor* A3(2), SAV: *S. avermitilis*, SGR: *S. griseus*)

Konservierte Kernregion (SCO2050-SCO5800)	Artspezifische Linker	Terminale Regionen (<SCO1100 und >SCO7600)
nicT2 (SAV_2333) *nur* (SCO_4180) *srnR* (SGR_4905) *srnQ* (SGR_4904) *sodF* (SGR_4906) *sodN* (SCO_5254) *sodN* (SAV_2988) *sodC* (SGR_5158)	*nikABCDE*-Operon SCO_6451–6455 *cbiX* SAV_6405 *cbiX* SCO_1858	*nikA*-Homolog (SGR_498) *nicT1* (SAV_560)

5. Zusammenfassung

Nickel ist einerseits lebenswichtig als Cofaktor in bakteriellen Enzymen eingebunden. Andererseits wirkt Nickel zum Teil schon in geringen Konzentrationen toxisch auf Zellen. Eine fein abgestimmte Regulierung der Aufnahme bzw. der Speicherung dieses Metalls ist deshalb notwendig. Es sind mehrere Enzyme bekannt, die Nickel enthalten, darunter die zuerst bei Streptomyceten nachgewiesene Nickel-Superoxiddismutase. Nickel dient zur Regulation der Expression dieser Proteine oft als Co-Regulator. Unter anderem induziert Nickel die Synthese der nickelhaltigen Urease, FeNi-Hydrogenase und NiSOD, während es die Eisenaufnahme und die FeSOD hemmt. Um Nickel verwenden zu können, müssen Zellen sensible Mechanismen entwickeln, die das Schwermetall binden, transportieren und an Zielproteine abgeben. Neben der Aufklärung der Regulation und Homöostase stellt sich aufgrund der enzymatischen Funktion auch die Frage nach der Rolle der NiSOD in der Resistenzvermittltung gegenüber Schwermetallen. Schwermetalle verursachen einerseits die Bildung zellschädigender reaktiver Sauerstoffspezies, die durch Superoxiddismutasen eliminiert werden können. Andererseits wird die NiSOD-Synthese nicht nur durch Nickel, sondern teilweise auch durch Schwermetalle induziert. Neben anderen untersuchten Resistenzfaktoren, die für die zum Teil hohe Resistenz gegenüber Schwermetallen bei aus anthropogen kontaminierten Böden isolierten Streptomyces-Stämmen verantwortlich sind, werden Superoxiddismutasen und besonders die NiSOD als resistenzvermittelnde Faktoren diskutiert.

Die NiSOD hat sich mit anderen bekannten SOD-Isoformen funktionell konvergent entwickelt, abgesehen von der einzigartigen Nickel-Koordination stellt die Ligandenumgebung wie auch in anderen SOD-Formen das Redoxpotential für die gleichartige Disproportionierung mit jeweils verschiedenen Cofaktoren bereit. Der Nickel koordinierende N-terminale Haken bildet jeweils eines der aktiven Zentren des Hexamers. Da dem Nickelhaken ähnelnde Ni-Komplexe bzw. Modelle bereits allein die Disproportionierung ausführen können, kann diese hochkonservierte Domäne als eine an ein Protein gebundene funktionelle Einheit betrachtet werden. Die Besonderheit des Cofaktors und dessen Einbindung in eine SOD, stellte die Frage nach der Evolution und Verbreitung im Organismenreich. Die vergleichende Analyse potentieller NiSOD-Gene verschiedener prokaryotischer Gruppen sowie eukaryotischer Algen zeigt evolutionäre Trends und trägt gleichzeitig zur Strukturaufklärung dieses einzigartigen Enzyms bei.

Abstract

While nickel is essential as cofactor of bacterial enzymes it acts toxic already at low concentrations. Therefore a fine tuned regulation of the uptake and storage of this metal is needed. Several nickel containing enzymes are known among which is a nickel containing superoxide dismutase first identified in streptomycetes. Nickel often serves as regulator for expression of these proteins. Amongst others nickel induces the synthesis of urease, FeNi-hydrogenase and NiSOD, whereas it inhibits iron uptake and synthesis of FeSOD. For the utilisation of nickel cells need to develop sensible mechanisms for binding, transport and incorporation of the heavy metal into the target protein. Besides elucidation of regulation and homeostasis the resistence mediating role of NiSOD based on its enzymatic activity was analysed. Heavy metals are involved in the generation of reactive oxygen species which are toxic to cells and could be eliminated by superoxide dismutases. The synthesis of NiSOD is induced not only by nickel but also in part by other heavy metals. We examined different resistance factors for heavy metal resistant streptomycetes isolated from anthropogenically contaminated soil. Superoxide dismutases and NiSOD in particular are discussed as resistance factor as well.

NiSOD has developed convergently to other known SOD isoforms. While nickel coordination indeed is unique the ligand environment is maintaining the redox potential for disproportionation of superoxide radicals in all SOD isoforms. The N-terminal nickel coordinating hook presents the active center subunit in the active homohexamer. Derivations of nickel hook sequences or maquettes are able to perform SOD function in vitro which classifies this highly conserved domain as a functional unit embedded in a protein backbone. Thus this SOD is well suited for analysis of evolution and distribution. The comparative analysis of putative NiSODs of different prokaryotic groups and of eukaryotic algae reveals evolutionary trends and, at the same time, contributes to structure analysis of this unique enzyme.

6. Literatur

Abbas, A., Edwards, C., 1989. Effects of metals on a range of *Streptomyces* species, Appl. and Environ. Microbiol. 55, 2030-2035.

Abbas, A.S., Edwards, C., 1990. Effect of metals on *Streptomyces coelicolor* growth and actinorhodin production, Appl. Environ. Microbiol. 56, 675-680.

Abou-Shanab, R.A.I., van Berkum, P., Angle, J.S., 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*, Chemosphere 68, 360-367.

Ahn, B.E., Cha, J., Lee, E.J., Han, A.R., Thompson, C.J., Roe, J.H., 2006. Nur, a nickel-responsive regulator of the Fur family, regulates superoxide dismutases and nickel transport in *Streptomyces coelicolor*, Mol. Microbiol. 59, 1848–1858.

Albarracin, V.H., Avila, A.L., Amoroso, M.J., Abate, C.M., 2008. Copper removal ability by *Streptomyces* strains with dissimilar growth patterns and endowed with cupric reductase activity, FEMS Microbiol Lett 288, 141–148.

Aleem, A., Isar, J. and Malik, A., 2003. Impact of long-term application of industrial wastewater on the emergence of resistance strains in *Azotobacter chroococcum* isolated from rhizosphere soil, Bioresour. Technol. 86, 7–13.

Amoroso, M.-J., Schubert, D., Mitscherlich, P., Schumann, P., Kothe, E., 2000. Evidence for high affinity nickel transporter genes in heavy metal resistant *Streptomyces spec.*, J. Basic Microbiol. 40, 295–201.

An, Y.J., Ahn, B.E., Roec, J.H., Chaa, S.S., 2008. Crystallization and preliminary X-ray crystallographic analyses of Nur, a nickel-responsive transcription regulator from *Streptomyces coelicolor*, Acta Cryst F64, 130–132.

An, Y.J., Ahn, B.E., Han, A.R., Kim, H.M., Chung, K.M., Shin, J.H., Cho, Y.B., Roe, J.H., Cha, S.S., 2009. Structural basis for the specialization of Nur, a nickel-specific Fur homolog, in metal sensing and DNA recognition, Nucl Acids Res 37, 103442–103451.

Anton, A., Große, C., Reißmann, J., Pribyl, T., Nies, D.H., 1999. CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34, J. Bacteriol. 181, 6876-6881.

Armougom, F., Moretti, S., Poirot, O., Audic, S., Dumas, P., Schaeli, B., Keduas, V., Notredame, C., 2006. Expresso: automatic incorporation of structural information in multiple sequence alignments using 3D-Coffee, Nucleic Acids Res. 34, W604–608.

Baath, E., Diaz-Ravina, M., Frostegard, A., Campbell, C.D., 1998. Effect of metal-rich sludge amendments on the soil microbial community, Appl. Environ. Microbiol. 64, 238-245.

Barondeau, D.P., Kassmann, C.J., Bruns, C.K., Tainer, J.A., Getzoff, E.D., 2004. Nickel superoxide dismutase structure and mechanism. Biochemistry 43, 8038–8047.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Benov, L.T., Fridovich, I., 1994. *Escherichia coli e*xpresses a copper- and zinc-containing superoxide dismutase J Biol Chem 269, 25310-25314.

Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harper, D.E., Harris, M.A., Quail, H., Kieser, D., Bateman, A., Brown, S., Chandra, G., Chen, C.W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C.H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M.A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B.G., Parkhill, J., Hopwood, D.A., 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417, 141-147.

Blindauer, C.A., Razi M.T., Campopiano D.J., Sadler P.J., 2007. Histidine ligands in bacterial metallothionein enhance cluster stability. J Biol Inorg Chem 12, 393-405.

Blokesch, M., Paschos, A., Theodoratou, E., Bauer, A., Hube, M., Huth, S., Böck, A., 2002. Metal insertion into NiFe-hydrogenases. Biochem. Soc. Transact. 30, 674–680.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Bryngelson, P.A., Arobo, S.E., Pinkham, J.L., Cabelli, D.E., Maroney, M.J., 2004. Expression, reconstitution, and mutation of recombinant *Streptomyces coelicolor* NiSOD, J. Am. Chem. Soc. 126, 460-461.

Chater, K.F., Biró, S., Lee, K.J., Palmer, T., Schrempf, H., 2010. The complex extracellular biology of *Streptomyces*. FEMS Microbiol Rev. 34, 171-198.

Chen, C.W., Huang, C.H., Lee, H.H., Tsai, H.H., Kirby, R., 2002. Once the circle has been broken: dynamics and evolution of *Streptomyces* chromosomes. TRENDS in Genetics 18, 522-529.

Choudhury, S.B., Lee, J.W., Davidson, G., Yim, Y.I., Bose, K., Sharma, M.L., Kang, S.O., Cabelli, D.E., Maroney, M.J., 1999. Examination of the nickel site structure and reaction mechanism in *Streptomyces seoulensis* superoxide dismutase. Biochemistry 23, 3744–3752.

Chung, H.J., Choi, J.H., Kim, E.J., Cho, Y.H., Roe, J.H., 1999a. Negative regulation of the gene for Fe-containing superoxide dismutase by an Ni-responsive factor in *Streptomyces coelicolor*. J. Bacteriol. 181, 7381–7384.

Chung, H.J., Kim, E.J., Suh, B., Choi, J.H., Roe, J.H., 1999b: Duplicate genes for Fe-containing superoxide dismutase in *Streptomyces coelicolor* A3(2). Gene 231, 87-93.

Claessen, D., de Jong, W., Dijkhuizen, L., Wösten, H.A., 2006: Regulation of *Streptomyces* development: reach for the sky!, Trends Microbiol. 14, 313-319.

Ciriolo, M.R., Civitareale, P., Carri, M.T., De Martino, A., Galiazzo, F., Rotilio, G., 1994. Purification and characterization of Ag, Zn-superoxide dismutase from *Saccharomyces cerevisiae* exposed to silver. J. Biol. Chem. 14, 25783–25787

Colquhoun, J.A., Heald, S.C., Li, L., Tamaoka, J., Kato, C., Horikoshi, K., Bull, A.T., 1998. Taxonomy and biotransformation activities of some deep-sea actinomycetes. Extremophiles 2, 269-277.

Compan, I., Touati, D., 1993. Interaction of six global transcription regulators in expression of manganese superoxide dimutase in *Escherichia coli* K12. J. Bacteriol. 175, 1687–1696.

Constant, P., Poissant, L., Villemur, R., 2008 . Isolation of *Streptomyces sp.* PCB7, the first microorganism demonstrating high-affinity uptake of tropospheric H_2, ISME Journal 2, 1066–1076.

Culotta, V.C., Joh, H., Lin, S., Slekar, K.H., Strain, J., 1995. A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. J. Biol. Chem. 270, 29991–29997.

Delorme, T.A., Gagliardi, J.V., Angle, J.S. and Chaney, R.L., 2001. Influence of the zinc hyperaccumulator *Thlapsi caerulescens* J. and C. Presl. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial populations. Can. J. Microbiol. 47, 773–776.

Derelle, E., Ferraz, C., Rombauts, S., Rouzé, P., Worden, et al., 2006. Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc. Natl. Acad. Sci. USA 103, 11647–11652.

Dimkpa, C.O., Svatoš, A., Dabrowska, P., Schmidt, A., Boland, W., Kothe, E., 2008a. Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in *Streptomyces* spp., Chemosphere 74, 19–25.

Dimkpa, C.O., Svatoš, A., Merten, D., Büchel, G., Kothe, E., 2008b. Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress, Can. J. Microbiol. 54, 163–172.

Dimkpa, C.O., Merten, D., Svatoš, A., Büchel, G., Kothe, E., 2009a. Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores, Soil Biol Biochem 41, 154–162.

Dimkpa, C.O., Merten, D., Svatos, A., Büchel, G., Kothe, E., 2009b. Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively, J Appl Microbiol 107, 1687–1696.

Dunlap, P.V., Steinman, H.M., 1986. Strain variation in bacteriocuprein superoxide dismutase from symbiotic *Photobacterium leiognathi*. J Bacteriol. 165, 393-398.

Dupont, C.L., Neupane, K., Shearer, J. and Palenik, B., 2008a. Diversity, function and evolution of genes coding for putative Ni-containing superoxide dismutases. Environ. Microbiol. 10, 1831–1843.

Dupont, S.L., Barbeau, K., Palenik, B., 2008b. Ni uptake and limitation in marine *Synechococcus s*trains, Appl. Environ. Microbiol. 74, 23–31.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucl. Acids Res. 32, 1792–1797.

Eitinger, T., Mandrand-Berthelot, M.A., 2000. Nickel transport systems in microorganisms. Arch Microbiol 173, 1–9.

Eitinger, T., 2004. *In vivo* production of active nickel superoxide dismutase from *Prochlorococcus marinus* MIT9313 is dependent on its cognate peptidase. J. Bacteriol. 186, 7821–7825.

Fiedler, A.T., Bryngelson, P.A., Maroney, M.J., Brunold, T.C., 2005. Spectroscopic and computational studies of Ni superoxide dismutase: Electronic structure contributions to enzymatic function. J. Am. Chem. Soc. 127, 5449–5462.

Fiedler, A.T., Brunold, T.C., 2007. Spectroscopic and computational studies of Ni^{3+} complexes with mixed S/N ligation: Implications for the active site of Nickel superoxide dismutase, Inorg. Chem. 46, 8511-8523.

Flärdh, K., Buttner, M.J., 2007: *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium, Nat Rev Microbiol. 7, 36-49.

Francisco, R., Alpoim, M.C., Morais, P.V., 2002. Diversity of chromium-resistant and -reducing bacteria in a chromiumcontaminated activated sludge. Appl. Microbiol. 92, 837–943.

Fridovich, I., 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. 64, 97–112.

Gale, E.M., Narendrapurapu, B.S., Simmonett, A.C., Schaefer, H.F., Harrop, T.C., 2010. Exploring the effects of H-bonding in synthetic analogues of nickel superoxide dismutase (Ni-SOD): Experimental and theoretical implications for protection of the Ni-SCys bond, Inorg. Chem. 2010 Jun 24. [Epub ahead of print]

Geletneky, J., Paul, M., Merten, D., Büchel, G., 2002. Impact of acid rock drainage in a discrete catchment area at the former uranium mining site Ronneburg (Germany). In: Nelson J.D., Cincilla W.A., Foulk, C.L., Hinshaw, L.L., Ketellaper, V. (eds) Tailings and mine waste, Proc, 9th Internat Conf on tailings and mine waste, Fort Collins, CO, USA, 67-74.

Geslin, C., Llanos, J., Prieur, D. and Jeanthon, C., 2001. The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. Res. Microbiol. 152, 901–905.

Grass, G., Grosse, C. and Nies, D.H., 2000. Regulation of the *cnr* cobalt and nickel resistance determinant from *Ralstonia sp. strain* CH34. J. Bacteriol. 182, 1390–1398.

Grass, G., Fan, B., Rosen, B.P., Lemke, K., Schlegel, H.-G., Rensing, C., 2001. NreB from *Achromobacter xylosoxidans* 31A is a nickel-induced transporter conferring nickel resistance. J. Bacteriol. 183, 2803-2807.

Grosse, C., Anton, A., Hoffmann, T., Franke, S., Schleuder, G., Nies, D.H., 2004. Identification of a regulatory pathway that controls the heavy-metal resistance system Czc via promotor *czcNp* in *Ralstonia metallidurans*. Arch. Microbiol. 182, 109–118.

Haferburg G., Schmidt, A., Reinicke, M., Merten, D., Büchel, G., Kothe, E., 2004. Adaptation to nickel tolerance or nickel resistance in streptomycetes isolated from contaminated soil samples. ISBN 987-21607-0-8.

Haferburg, G., Kothe, E., 2007. Microbes and metals: interactions in the environment, J Basic Microbiol 47, 453–467.

Haferburg, G., Reinicke, M., Merten, D., Büchel, G. and Kothe, E., 2007. Microbes adapted to acid mine drainage as source for strains active in retention of aluminium or uranium. J. Geochem. Expl. 92, 196–204.

Haferburg, G., Kloess, G., Schmitz, W., Kothe, E., 2008. "Ni-struvite" – A new biomineral formed by a nickel resistant *Streptomyces acidiscabies*. Chemosphere 72, 517–523.

Haiser, H.J., Yousef, M.R., Elliot, M.A., 2009. Cell wall hydrolases affect germination, vegetative growth, and sporulation in *Streptomyces coelicolor*. J Bacteriol. 191, 6501-6512.

Hahn, J.-S., Oh, S.-Y., Roe, J.-H., 2000. Regulation of the *furA* and *catC* operon, encoding a ferric uptake regulator homolog and catalase-peroxidase, respectively, in *Streptomyces coelicolor A3(2)*. J. Bacteriol. 275, 3767–3774.

Herbst, R.W., Guce, A., Bryngelson, P.A., Higgins, K.A., Ryan, K.C., Cabelli, D.E., Garman, S.C., Maroney, M.J., 2009. Role of conserved Tyrosine residues in NiSOD catalysis: A case of convergent evolution, Biochemistry 48*,* 3354–3369.

Hery, M., Nazaret, S., Jaffre, Z., Normand, P., Navarro, E., 2003. Adaptation to nickel spiking of bacterial communities in neocaledonian soil. Environm. Microbiol. 5, 3–12.

Hopwood, D.A., 2006. Soil to genomics: the *Streptomyces* chromosome. Annu. Rev. Genet. 40, 2006.

Hsiao, N., Kirby, R., 2007. Comparative genomics of *Streptomyces avermitilis*, *Streptomyces cattleya*, *Streptomyces maritimus* and *Kitasatospora aureofaciens* using a *Streptomyces coelicolor* microarray system, Antonie Van Leeuwenhoek 93, 1-25.

Hua, X., Li, J., Li, J., Zhang, L., Cui, Y., 2009: Selective inhibition of the cyanobacterium, *Microcystis*, by a *Streptomyces sp.*. Biotechn. Lett. 31, 1531-1535.

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W.W., Sessitsch, A., 2004. Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. Appl. Environ. Microbiol. 70, 2667-2677.

Idris, R., Kuffner, M., Bodrossy, L., Puschenreiter, M., Monchy, S., Wenzel, W.W., Sessitsch, A., 2006. Characterization of Ni-tolerant methylobacteria associated with the hyperaccumulating plant *Thlaspi goesingense* and description of *Methylobacterium goesingense* sp. nov.. Syst. Appl. Microbiol. 29, 634-644.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M., Omura, S., 2003. Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. Nat Biotechnol 21, 526-531.

Imlay, K.R.C., Imlay, J.A., 1996. Cloning and Analysis of *sodC*, Encoding the Copper-Zinc Superoxide Dismutase of *Escherichia coli*. J Bacteriol. 178, 2564–2571.

Jakimowicz, D., 2007: Chromosome segregation and cell division during the growth and differentiation of *Streptomyces*, Postepy Hig Med Dosw. 61, 565-575.

Kaluarachchi, H., Chung, K.C.C., Zamble, D.B., 2010. Microbial nickel proteins, Nat Prod Rep 27, 681–694.

Kho, D.H., Yoo, S.B., Kim, J.S., Kim, E.J., Lee, J.K., 2004. Characterization of Cu- and Zn-containing superoxide dismutase of *Rhodobacter sphaeroides*. FEMS Microbiol Lett. 234, 261-267.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical *Streptomyces* genetics. The John Innes foundation, Norwich, UK.

Kim, F.J., Kim, H.P., Hah, Y.C., Roe, J.H., 1996. Differential expression of superoxide dismutases containing Ni and Fe/Zn in *Streptomyces coelicolor*. Eur J Biochem. 241, 178-185.

Kim, E.J., Chung, H.J., Suh, B., Hah, Y.C., Roe, J.H., 1998a. Transcriptional and post-transcriptional regulation by nickel of *sodN* gene encoding nickel-containing superoxide dismutase from *Streptomyces coelicolor* Müller. Mol. Microbiol. 27, 187–195.

Kim, E.J., Chung, H.J., Suh, B., Hah, Y.C., Roe, J.H., 1998b. Expression and regulation of the *sodF* gene encoding iron and zinc-containing superoxide dismutase in *Streptomyces coelicolor* Müller. J. Bact. 180, 2014–2020.

Kim, J.S., Jang, J.H., Lee, J.W., Kang, S.O., Kim, K.S., Lee, J.K., 2000. Identification of *cis* site involved in nickel responsive transcriptional repression of *sodF* gene coding for Fe- and Zn-containing superoxide dismutase of *Streptomyces griseus*. Biochim. Biophys. Acta 1493, 200–207.

Kim, I.K., Yim, Y.I., Kim, J.M., Lee, J.W., Yim, H.S., Kang, S.O., 2003a. CbiX-homologous protein (CbiXhp), a metal-binding protein, from *Streptomyces seoulensis* is involved in expression of nickel-containing superoxide dismutase. FEMS Microbiol. Lett. 228, 21–26.

Kim, J.S., Kang, S.O., Lee, J.K., 2003b. The protein complex composed of nickel-binding SrnQ and DNA binding motifbearing SrnR of *Streptomyces griseus* represses *sodF* transcription in the presence of nickel. J. Biol. Chem. 278, 18455–18463.

Kirby, T.W., Lancaster, J.R.Jr., Fridovich, I., 1981: Isolation and characterization of the iron-containing superoxide dismutase of *Methanobacterium bryantii*. Arch. Biochem. Biophys. 210, 140-148.

Knoblauch, C., Sahm, K., Jørgensen, B.B., 1999. Psychrophilic sulfate-reducing bacteria isolated from permanently cold arctic marine sediments: description of *Desulfofrigus oceanense gen. nov., sp. nov., Desulfofrigus fragile sp. nov., Desulfofaba gelida gen. nov., sp. nov., Desulfotalea psychrophila gen. nov., sp. nov. and Desulfotalea arctica sp. nov*. Int J Syst Bacteriol 49, 1631-1643.

Koch, S., 2010. Regulation einer nickelhaltigen Superoxid-Dismutase in schwermetall-resistenten Streptomyceten. Diplomarbeit an der FSU Jena, Institut für Mikrobielle Phytopathologie.

Kothe, E., Dimkpa, C., Haferburg, G., Schmidt, A., *Schmidt, A., Schütze, E., 2010. Streptomycete heavy metal resistance: Extracellular and intracellular mechanisms, In: Sherameti. I., Varma, A. (eds.), Soil Heavy Metals, Kapitel 10, Verlag Springer Berlin Heidelberg, ISBN 978-3-642-02436-8.

Komatsu, M., Tsuda, M., Omura, S., Oikawa, H., Ikeda, H., 2008. Identification and functional analysis of genes controlling biosynthesis of 2-methylisoborneol, Proc Natl Acad Sci USA 105, 7422-7427.

Leclere, V., Boiron, P., Blondeau, R., 1999. News and notes: diversity of superoxide-dismutases among clinical and soil isolates of *Streptomyces* species. Curr. Microbiol. 39, 365–368.

Lee, H.I., Lee, J.W., Yang, T.C., Kang, S.O., Hoffman, B.M., 2010. ENDOR and ESEEM investigation of the Ni-containing superoxide dismutase. J Biol Inorg Chem 15, 175-182.

Lee, J.W., Roe, J.H. and Kang, S.O., 2002. Nickel-containing superoxide dismutase. Methods Enzymol. 349, 90–101.

Lischke, U., 2008. Adaption an Nickelstress von *Streptomyces acidiscabies* E13, Diplomarbeit an der FSU Jena, Institut für Mikrobielle Phytopathologie.

Lopez-Maury, L., Garcia-Dominguez, M., Florencio, F.J., Reyes, J.C., 2002. A two component signal transduction system involved in nickel sensing in the cyanobacterium *Synechocystis* sp. PCC 6803. Mol. Microbiol. 43, 247-256.

Madigan, M.T., Martinko, J.M., Parker, J., 2000. Mikrobiologie, Spektrum Verlag Heidelberg.

Martin, M., Byers, B.R., Olson, M.O.J., Salin, M.L., Arceneaux, J.E.L., Tolbert, C., 1986. A *Streptococcus mutans* superoxide dimutase that is active with either manganese or iron as cofactor. J. Biol. Chem. 261, 9361–9367.

Meier, B., Barra, D., Bossa, F., Calabrese, L., Rotilio, G., 1982. Synthesis of either Fe- or Mn-superoxide dimutase with an apparently identical protein moiety by an anaerobic bacterium dependent on the metal supplied, J. Biol. Chem. 257, 13977-13980.

Meier, B., Sehn, A.P., Sette, M., Paci, M., Desideri, A., Rotilio, G., 1994. *In vivo* incorporation of cobalt into *Propionibacterium shermanii* superoxide dismutase. FEBS Lett. 348, 283–286.

Mengoni, A., Barzanti, R., Gonnelli, C., Gabbrielli, R., Bazzicalupo, M., 2001. Characterization of nickel-resistant bacteria isolated from serpentine soil. Environ. Microbiol. 3, 691–698.

Mengoni, A., Grassi, E., Barzanti, R., Biondi, E.G., Gonnelli, C., Kim, C.K., Bazzicalupo, M., 2004. Genetic diversity of bacterial communities of serpentine soil and of rhizosphere of the nickel-hyperaccumulator plant *Alyssum bertolonii*. Microb. Ecol. 48, 209–217.

Mergeay, M., Nies, D., Schlegel, H.D., Gerits, J., Charles, P., van Gijsegem, F., 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162, 328-334.

Mergeay, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., van der Lelie, D., Wattiez, R., 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals towards a catalogue of metal-responsive genes. FEMS Microbiol. Rev. 27, 385-410.

Merten, D., Kothe, E., Büchel, G. 2004. Studies on microbial heavy metal retention from uranium mine drainage water with special emphasis on rare earth elements. Mine Water Environ. 23, 34-43.

Monciardini, P., Sosio, M., Cavaletti, L., Chiocchini, C., Donadio, S., 2002. New PCR primers for the selective amplification of 16S rDNA from different groups of actinomycetes. FEMS Microbiol. Ecology 42, 419-429.

Morita, E.H., Wakamatsu, M., Kawamoto, S., Nishiyama, Y., Hayashi, H., 2003. Studies on the protein-DNA complex formation between the cyanobacterial transcription factors, SmtB and its homologues, functioning as zinc-ion sensors and the recognition DNA sequences. Nucleic Acids Res Suppl 3, 203-204.

Mullins, C.S., Grapperhaus, C.A., Frye, B.C., Wood, L.H., Hay, A.J., Buchanan, R.M., Mashuta, M.S., 2009. Synthesis and sulfur oxygenation of a (N_3S)Ni complex related to nickel-containing superoxide dismutase, Inorg Chem 48, 9974-9976.

Neupane, K.P., Shearer, J., 2006. The influence of amine/amide versus bisamide coordination in nickel superoxide dismutase. Inorg. Chem. 45, 10552–10566.

Neupane, K.P., Gearty, K., Francis, A., Shearer, J., 2007. Probing variable axial ligation in nickel superoxide dismutase utilizing metallopeptide-based models: Insight into the superoxide disproportionation mechanism. J Am Chem Soc 129, 14605-14618.

Nies, D.H., Mergeay, M., Barbel, F., Schlegel, H.D., 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. 169, 4865-4868.

Nies, D.H., 2003. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 27, 313-339.

Noel-Georis, I., Vallaeys, T., Chauvaux, R., Monchy, S., Falmagne, P., Mergeay, M., Wattiey, R., 2004. Global analysis of the *Ralstonia metallidurans* proteome: prelude for the large-scale study of heavy metal response. Proteomics 4, 151-179.

Olafson, R.W., McCubbin, W.D., Cyril, M.K., 1988. Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from a *Synechococcus sp.* cyanobacterium. Biochem. J. 251, 691-699.

Pal, A., Dutta, S., Mukherjee, P.K., Paul, A.K., 2005. Occurence of heavy metal-resistance in microflora from serpentine soil of Andaman. J. Basic Microbiol. 45, 207–218.

Palenik, B., Brahamsha, B., Larimer, F.W., Land, M., Hauser, L., Chain, P., Lamerdin, J., Regala, W., Allen, E.E., McCarren, J., Paulsen, I., Dufresne, A., Partensky, F., Webb, E.A. and Waterbury, J., 2003. The genome of a motile marine *Synechococcus*. Nature 424, 1037–1042.

Palenik, B., Ren, Q., Dupont, C.L., Myers, G.S., Heidelberg, J.F., Badger, J.H., Madupu, R., Nelson, W.C., Brinkac, L.M., Dodson, R.J., Durkin, A.S., Daugherty, S.C., Sullivan, S.A., Khouri, H., Mohamoud, Y., Halpin, R., Paulsen, I.T., 2006. Genome sequence of *Synechococcus* CC9311: Insights into adaptation to a coastal environment. PNAS 103, 13555–13559.

Pelmenschikov, V., Siegbahn, P.E.M., 2006. Nickel superoxide dismutase reaction mechanism studied by hybrid density functional methods. J. Am. Chem. Soc. 128, 7466–7475.

Pimentel-Elardo SM, Scheuermayer M, Kozytska S, Hentschel U., 2009. *Streptomyces axinellae* sp. nov., isolated from the Mediterranean sponge *Axinella polypoides* (Porifera). Int J Syst Evol Microbiol 59, 1433-1437.

Polti, M.A., Amoroso, M.J., Abate, C.M., 2007. Chromium(VI) resistance and removal by actinomycete strains isolated from sediments. Chemosphere 67, 660–667.

Priya, B., Premanandh, J., Dhanalakshmi, R.T., Seethalakshmi, T., Uma, L., Prabaharan, D., Subramanian, G., 2007. Comparative analysis of cyanobacterial superoxide dismutases to discriminate canonical forms. BMC Genomics 8, 1–10.

Rabus, R., Ruepp, A., Frickey, T., Rattei, T., Fartmann, B., Stark, M., Bauer, M., Zibat, A., Lombardot, T., Becker, I., Amann, J., Gellner, K., Teeling, H., Leuschner, W. D., Glöckner, F.-O., Lupas, A. N., Amann R., Klenk H.-P., 2004. The genome of *Desulfotalea psychrophila*, a sulfatereducing bacterium from permanently cold Arctic sediments. Environm Microbiol 6, 887–902.

Rajapaksha, R.M., Tobor-Kaplon, M.A. and Baath, E., 2004. Metal toxicity affects fungal and bacterial activities in soil differently. Appl. Environ. Microbiol. 70, 2966–2973.

Ravel, J., Schrempf, H., Hill, R.T., 1998. Mercury resistance is encoded by transferable giant linear plasmids in two Chesapeake Bay *Streptomyces* strains. Appl. Environ. Microbiol. 64, 3383-3388.

Ravel, J., Wellington E.M.H., Hill R.T., 2000. Interspecific transfer of *Streptomyces* giant linear plasmids in sterile amended soil microcosms. Appl. Environ. Microbiol. 66, 539-534.

Ravel, J., DrRuggiero, J., Robb, F.T., Hill, R.T., 2000. Cloning and sequence analysis of the mercury resistance operon of *Streptomyces sp. strain* CHR28 reveals a novel putative second regulatory gene. J. Bacteriol. 182, 2345-2349.

Ray, G. and Husain, S.A., 2002. Oxidants, antioxidants and carcinogenesis. Indian J. Exp. Biol. 40, 1213–1232.

Raytapadar, S., Datta, R., Paul, A.K., 1995. Effects of some heavy metals on growth, pigment and antibiotic production by *Streptomyces galbus*. Acta Microbiol. Immunol. Hung. 42, 171-177.

Roane, T.M., Kellogg, S.T., 1996. Characterization of bacterial communities in heavy metal contaminated soils. Can. J. Microbiol. 42, 593–603.

Robinson, N.J., Whitehall, S.K., Cavet, J.S., 2001. Microbial metallothioneins. Adv. Microb. Physiol. 44, 183-213.

Rodionov, D.A., Hebbeln, P., Gelfand, M.S., Eitinger, T., 2006. Comparative and functional genomic analysis of prokaryotic nickel and cobalt uptake transporters: Evidence for a novel group of ATP-binding cassette transporters. J. Bacteriol. 188, 317–327.

Rodríguez, F., Derelle, E., Guillou, L., Le Gall, F., Vaulot, D., Moreau, H., 2005. Ecotype diversity in the marine picoeukaryote *Ostreococcus* (*Chlorophyta*, *Prasinophyceae*). Environ. Microbiol. 7, 853–859.

Ryan, K.C, Johnson, O.E., Cabelli, D.E., Brunold, T.C., Maroney, M.J., 2010. Nickel superoxide dismutase: structural and functional roles of Cys2 and Cys6, J Biol Inorg Chem 15, 795-807.

Schmidt, A., Haferburg, G., Sineriz, M., Merten, D., Büchel, G., Kothe, E., 2005. Heavy metal resistance mechanisms in actinobacteria for survival in AMD contaminated soils. Chemie Erde 65, 131–144.

*Schmidt, A., Schmidt, A., Haferburg, G., Kothe, E., 2007. Superoxide dismutases of heavy metal resistant streptomycetes, J. Basic Microbiol 47, 56–62.

Schmidt, A., Haferburg, G., *Schmidt, A., Kothe, E., 2007. Heavy metal resistance in actinobacteria of the former uranium mining area near Ronneburg. In: Proceedings des Internationalen Bergbausymposiums WISMUT 2007, Stilllegung und Revitalisierung von Bergbaustandorten zur nachhaltigen Regionalentwicklung, Wismut GmbH.

Schmidt, M., Zahn, S., Carella, M., Öhlenschläger, O., Görlach, M., Kothe, E., Weston, J., 2008. Solution structure of a functional biomimetic and mechanistic implications for nickel superoxide dismutases. Chem. Bio. Chem. 9, 11–13.

*Schmidt, A., Gube, M., Schmidt, A., Kothe, E., 2009. *In silico* analysis of nickel containing superoxide dismutase evolution and regulation. J Basic Microbiol 49, 109–118.

Schmidt, A., Haferburg, G., *Schmidt, A., Lischke, U., Merten, D., Ghergel, F., Büchel, G., Kothe, E., 2009. Heavy metal resistance to the extreme: *Streptomyces* strains from a former uranium mining area. Chemie der Erde 69, 35–44.

Schmidt, A., Hagen, M., Schütze, E., *Schmidt, A., Kothe, E., 2010. Screening for potential Metallothioneins and Metallohistins in Actinobacteria. J Basic Microbiol [accepted]

Shallari, S., Schwartz, C., Hasko, A., Morel, J.L., 1998. Heavy metals in soils and plants of serpentine and industrial sites of Albania. Sci. Total Environ. 209, 133–142.

Shearer, J. and Long, L.M., 2006. A nickel superoxide dismutase maquette that reproduces the spectroscopic and functional properties of the metalloenzyme. Inorg. Chem. 45, 2358–2360.

Shearer, J., Dehestani, A., Abanda, F., 2008. Probing variable amine/amide ligation in $NiIIN_2S_2$ complexes using sulfur K-edge and nickel L-edge X-ray absorption spectroscopies: Implications for the active site of nickel superoxide dismutase. Inorg Chem 47, 2649-2660.

Shearer, J., Neupane, K.P., Callan, P.E., 2009. Metallopeptide based mimics with substituted histidines approximate a key hydrogen bonding network in the metalloenzyme nickel superoxide dismutase. Inorg Chem 48, 10560–10571.

Shevchenko, V., Lisitzin, A., Vinogradova, A., Stein, R., 2003. Heavy metals in aerosols over the seas of the Russian arctic. Sci. Total Environ. 306, 11-25.

Silaghi-Dumitrescu, R., 2009. Superoxide interaction with nickel and iron superoxide dismutases. J Mol Graphics Modelling 28, 156–161.

Siñeriz, M.L., Kothe, E., Abate, C.M., 2009. Cadmium biosorption by *Streptomyces* sp. F4 isolated from former uranium mine. J Basic Microbiol, 49, S55–S62.

Smylla, A., Mroczkowska-Badner, E., 1991. Influence of cadmium ions on *Streptomyces* strains. Acta Microbiol. Pol. 40, 51-58.

Snavely, M.D., Florer J.B., Miller, C.G., Maguire, M.E., 1989. Magnesium transport in *Salmonella typhimurium*: Mg^{2+} transport by the CorA, MgtA, and MgtB systems. J. Bacteriol. 171, 4761-4766.

Sontag, B., Gerlitz, M., Paululat, T., Rasser, H.F., Grün-Wollny, I., Hansske, F.G., 2006. Oxachelin, a novel iron chelator and antifungal agent from *Streptomyces sp.* GW9/1258. J Antibiot (Tokyo) 59, 659-663.

Stohs, S.J., Bagchi, D., 1993. Oxidative mechanisms in the toxicity of metal ion. Free Radical Biol. Med. 18, 321–336.

Szilagyi, R.K., Bryngelson, P.A., Maroney, M.J., Hedman, B., Hodgson, K.O., Solomon, E.I., 2004. S K-edge X-ray absorption spectroscopic investigation of the Ni-containing superoxide dismutase active site: new structural insight into the mechanism. J. Am. Chem. Soc. 126, 3018–3019.

Tietze, D., Breitzke, H., Imhof, D., Kothe, E., Weston, J., Buntkowsky, G., 2009. New insight into the mode of action of nickel superoxide dismutase by investigating metallopeptide substrate models. Chemistry 15, 517–523.

Toledo, G. Palenik, B., 1997. *Synechococcus* diversity in the California current as seen by RNA Polymerase (*rpoC1*) gene sequences of isolated strains. Appl Environm Microbiol 63, 4298–4303.

Van Nostrand, J.D., Khijniak, T.V., Gentry, T.J., Novak, M.T., Sowder, A.G., Zhou, J.Z., Bertsch, P.M., Morris, P.J., 2007. Isolation and characterization of four gram-positive nickel-tolerant microorganisms from contaminated sediments. Microb. Ecol. 53, 670-682.

Watt, R.K., Ludden, P.W., 1999. Nickel-binding proteins. Cell Mol. Life Sci. 56, 604–625.

Würges, J., Lee, J., Yim, Y., Yim, H., Kang, S., Carugo, K.D., 2004. Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. Proc. Natl. Acad. Sci. USA 101, 8569–8574.

Xiao J, Wang Y, Luo Y, Xie SJ, Ruan JS, Xu J., 2009: *Streptomyces avicenniae sp. nov.*, a novel actinomycete isolated from the rhizosphere of the mangrove plant *Avicennia mariana*. Int J Syst Evol Microbiol. 59, 2624-2628.

Yoshida M., Yoshida T., Takashima Y., Kondo R., Hiroishi S., 2005: Genetic diversity of the toxic cyanobacterium *Microcystis* in Lake Mikata. Environ Toxicol. 20, 229-234.

Youn, H.-D., Kim, E.-J., Roe, J.-H., Hah, Y.C., Kang, S.-O., 1996a. A novel nickel-containing superoxide dismutase from *Streptomyces spp.*. Biochem. J. 318, 889–896.

Youn, H.-D., Youn, H., Lee, J.-W., Yim, Y.-I., Lee, J.K., Hah, Y.C., Kang, S.-O., 1996b. Unique isoenzymes of superoxide dismutase in *Streptomyces griseus*. Arch. Biochem. Biophys. 334, 341–348.

Zeien, H., Bruemmer, G.W., 1989. Chemische Extraktionen zur Bestimmung von Schwermetallbindungsformen in Boeden. Mitt. Dtsch. Bodenkundl. Gesellsch. 59, 505–510.

Zhang, Z., Wang, Y., Ruan, J., 1997. A proposal to revive the genus *Kitasatospora* (Omura, Takahashi, Iwai, and Tanaka 1982). Int. J. Syst. Bacteriol. 47, 1048–1054.

7. Abkürzungsverzeichnis

AA: Aminosäuren

bp: Basenpaare

CuZnSOD: Kupfer-Zink-haltige Superoxiddismutase

C: Aminosäure Cystein

Cys: Aminosäure Cystein

Da: Dalton, biochemische Maßeinheit, Synonym für die atomare Masseneinheit (u), festgelegt auf genau 1/12 der Masse des Kohlenstoff-Isotops ^{12}C und entspricht damit annähernd der Masse des Wasserstoff-Isotops ^{1}H (1,00794 u).

DNA: Desoxyribonukleinsäure

EMSA: Electromobility Shift Assay zum Nachweis DNA-bindender Proteine

ESI-MS: Elektrosprayionisation-Massenspektronomie, u.a. zur Sequenzierung von Proteinen

et al.: *et alii* (lateinisch „und andere")

FeSOD: Eisenhaltige Superoxiddismutase

FeZnSOD: Eisen-Zink-haltige Superoxiddismutase

H: Aminosäure Histidin

His: Aminosäure Histidin

MnSOD: Mangan-haltige Superoxiddismutase

NiSOD: Nickel-haltige Superoxiddismutase

NiSODox: Enzym im oxidierten Zustand

NiSODred: Enzym im reduzierten Zustand

nt: Nukleotide

ORF: offener Leserahmen, potentiell proteinkodierende Gensequenz

RNAP: RNA-Polymerase

SOD: Superoxiddismutase

8. Verzeichnis der Abbildungen und Tabellen

9. Eigenständigkeitserklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation mit dem Thema

„Nickel-Superoxiddismutasen von schwermetallresistenten Streptomyceten"

selbstständig und ohne unerlaubte fremde Hilfe angefertigt habe. Es wurden von mir ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen. Eine Promotionsarbeit über dieses Thema liegt noch nicht vor.

Astrid Schmidt
Jena, den 30. August 2010

Danksagung

Die vorliegende Dissertation wäre ohne die Unterstützung einer Vielzahl von Menschen nicht gelungen.

In erster Linie gilt mein Dank meinen beiden geliebten Mädchen Helene und Lisa, die durch ihre Verspieltheit und Freude eine stets willkommene Ablenkung vom Ernst der Arbeit sind - und meinem Freund fürs Leben Andre, der ebenfalls immer für gelungene Abwechslung im Alltagstrott sorgt.

Meiner ganzen Familie möchte ich für alle Unterstützung danken, die sie mir gegeben haben. Danke an Mama Martina und Papa Bernd, Irina, Max und Goran, Oma Rosa und Opa Walter, Opa Hans und Renate, Katrin, Julia, Jan und Gerald und an die Familie von Andre, sowie Anna und Kurt.

Weiterer Dank gilt meiner Arbeitsgruppe und allen Mitarbeitern am Institut für Mikrobielle Phytopathologie der FSU Jena, insbesondere meiner Betreuerin Frau Prof. Erika Kothe, die mir auch während meiner Elternzeit die Weiterführung der Doktorarbeit ermöglichte. Mein Dank gilt auch allen Beteiligten des SFB 436 „Metallvermittelte Reaktionen nach dem Vorbild der Natur" und den Mitarbeitern am MPI für Chemische Ökologie für die Zusammenarbeit.

Ein großer Dank geht an meine Freunde Rebecca, Naomi und David, Saskia, Svea, Jan und Matthias, Antje und Moritz, allen Freunden unserer Streuobstwiese Bergsulza und vielen weiteren für die vielen erholsamen und lustigen Nachmittage auf Spielplätzen, im Garten, auf der Wiese und überhaupt in Jena.

Nicht zuletzt möchte ich allen Tieren danken, meine Arbeit für den Tierschutz motivierte mich besonders, diese Doktorarbeit fertig zu stellen.